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Modulation of small RNA silencing by cross-generational signaling in *C. elegans*

A dissertation presented

by

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to

The Division of Medical Sciences

In partial fulfillment of the requirements

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Doctor of Philosophy

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Modulation of small RNA silencing by cross-generational signaling in *C. elegans*

Abstract

Organisms are constantly challenged by the surrounding environment and alter their physiology accordingly. Some environment-induced changes in one generation are inherited in the offspring, and this long-lasting memory of parental experience has gained a lot of attention recently due to its implications in the organism's development and health. One example is transmission of RNAi-induced silencing from parents to progeny in *C. elegans*. Although this phenomenon has been known for more than a decade, the parental contribution to RNAi inheritance is still unclear. Here, we show that the nuclear hormone receptor DAF-12 mediates a cross-generational signaling that regulates RNAi in zygotes. Pol II ChIP-qPCR revealed that normally, DAF-12 enhances transcriptional repression induced by RNAi. Mutant analysis demonstrated that the role of DAF-12 in RNAi is distinct from its function in developmental timing or heterochronic pathways. Surprisingly, DAF-12 acts in mothers to alter the RNAi efficiency in zygotes, indicating the presence of mother-to-offspring, DAF-12-dependent signals that enhance RNAi in zygotes. Considering the previous studies showing that the function of DAF-12 is determined by environmental cues, we tested and found that the role of DAF-12 in RNAi enhancement in zygotes depends on the environmental cues presented to mothers during their development. These results demonstrate a novel role of DAF-12 as a modulator of

RNAi and its contribution to cross-generational signaling. Moreover, the findings imply a potential interaction between environmental conditions and small RNA pathways.

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To my family and friends

who believed in me

more than I believed in myself

ACKNOWLEDGEMENTS

On the cover of my thesis, I am listed as the sole author. However, if permitted, I would have filled the title page with endless lines of names of people who made this work possible—I might have needed a second title page.

First and foremost, I would like to thank Susan for her support and guidance throughout my years in her lab. I owe her my intellectual growth and invaluable experiences in the last four years. Everything I learned, including scientific thinking process and presentation skills, will be the solid basis of my future careers. I am heavily indebted to my dissertation committee, Dr. Keith Blackwell, Dr. Norbert Perrimon, and Dr. Piali Sengupta, for their incredibly helpful suggestions and comments on my work. Dr. Blackwell was the committee chair of which every student dreams. He would remain my role model for a mentor. I also owe huge thanks to my lab mates who created the best environment to do science. Researchers in the worm (and warm) labs at Harvard University—especially, Dr. Yun Zhang, Dr. Craig Hunter, Dr. Jimmy Zhuang, and Dr. Linjiao Luo—were invaluable sources whenever I needed to grasp in-depth background or learn new techniques.

In addition to brilliant scientists I have interacted with, non-scientists around me also made a significant contribution to my work. I have been extremely fortunate to have my life coaches, Kiyoung Oh and Sangwook Jung, who have never doubted my potential, offered priceless advice and shared with me their insights about the future. Besides all the exciting findings I described in this thesis, I discovered and

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CHAPTER I

Background

Transmission of environmental information from parents to offspring

In the past few decades, studies have shown a strong causative relationship between parental experience of environmental conditions and the abnormal phenotypes of their offspring without any genetic mutations. Both invertebrates and vertebrates exhibit such inheritance, and a range of environmental factors can act as a trigger. This unconventional inheritance began to receive a lot of attention due to its implications for human health, particularly obesity and heart disease. The identities of the molecules mediating the transmission of the environmental effect to offspring are under rigorous investigation. Epigenetic molecules, such as DNA methylation, small non-coding RNAs and histone modifications, have been suggested as strong candidates, but most studies have uncovered a correlation, not causality, between alteration in non-genetic molecules and phenotypic changes. Here, I summarize the cases where transient environmental conditions during the parental generation affect the subsequent generation. Following sections are the excerpts (with some modifications) from the review article "Hunting for Darwin's gemmules and Lamarck's fluid: transgenerational signaling and histone methylation" submitted to *BBA Gene Regulatory Mechanisms*.

Food and nutrients

Studies exploiting historical statistics and health records of individuals in Sweden have shown a strong correlation between food availability for grandfathers just prior to the

onset of puberty, and the longevity and risks for cardiovascular and metabolic diseases of their descendants (KAATI *et al.* 2002, 2007). When paternal grandfathers received plentiful food during the slow growth period prior to puberty, the descendants had a four-fold greater incidence of mortality from diabetes (KAATI *et al.* 2002). Another frequently cited example for inherited effects with humans is the Dutch Hunger Winter during World War II. A food embargo imposed by the Nazis on the uncooperative Dutch citizens resulted in a devastating famine during the winter of 1944-1945 (ZEE 1998; SCHULZ 2010). Although the famine lasted approximately five months, studies found that people who were exposed to the famine prenatally had a high incidence of health defects more than 50 years later. They were more likely to develop adult-onset diseases and experience a decline in their cognitive ability earlier, compared to their unexposed siblings (ROSEBOOM *et al.* 2006; DE ROOIJ *et al.* 2010; SCHULZ 2010).

To understand how a short period of starvation could induce health problems across generations, researchers developed and examined model organisms. Dunn and Bale (2009) reported that a high fat diet given to mice during pregnancy produced offspring (F1) with high body weight and low insulin sensitivity (DUNN and BALE 2009). These F1 animals were fed normally during development and pregnancy, yet their offspring (F2) also exhibited problems with insulin insensitivity (DUNN and BALE 2009).

Non-genetic inheritance can be induced from fathers and grandfathers. In a study by Fullston and colleagues, male mice fed a high-fat diet for ten weeks before mating produced F1 offspring with impaired glucose tolerance and insulin sensitivity (FULLSTON *et al.* 2013). Interestingly, obesity- and diabetes-related complications were more severe in F1 females than in F1 males, suggesting a gender bias (FULLSTON *et al.* 2013). In the

next generation, F2 females from the F1 males exhibited high body fat and insulin resistance (FULLSTON *et al.* 2013). The phenotypes observed in health and longevity were matched by changes in the expression of metabolic genes. For example, Ng and colleagues found that mice fed a high fat diet produced F1 female offspring with altered expression of genes important for insulin function and glucose metabolism (NG *et al.* 2010). Similarly, when male mice experienced a diet high in sugar and low in protein, the offspring had altered expression of genes for lipid and cholesterol metabolism (CARONE *et al.* 2010). Together, the amounting data suggest that non-genetic inheritance is responsible for alterations in metabolism across generations.

Toxins

Exposure to vinclozolin is one of the best-characterized examples of non-genetic inheritance from an environmental compound (ANWAY *et al.* 2005). Vinclozolin is a fungicide that interrupts endocrine signaling pathways and has long-lasting effects on future generations. When a gestating female rat is exposed to vinclozolin, her male F1 progeny exhibit a reduction in the number and motility of their sperm, leading to defects in reproduction that persist until the F4 generation (ANWAY *et al.* 2005). Other environmental compounds including di-(2-ethylhexyl) phthalate (a chemical component of plastics), pesticides, and jet fuel are also known to cause reproductive defects in progeny of the parents who are directly exposed to these chemicals (MANIKKAM *et al.* 2012; DOYLE *et al.* 2013). This finding highlights the abiding dangers of these chemicals for the ecosystem and human health.

Drosophila has provided good examples of inheritance of drug-induced phenotypes. Pentylenetetrazole is a GABA antagonist that induces epileptic seizures (KUPFERBERG 2001). A study using *Drosophila* as a model for epileptogenesis discovered that pentylenetetrazole treatment alters expression of genes in the central nervous system (MOHAMMAD *et al.* 2009). Strikingly, grandchildren of pentylenetetrazole-treated male *Drosophila* showed expression profiles that were similar to those of the P0 flies, indicating the potency of this chemical in subsequent generations (SHARMA and SINGH 2009).

Stern *et al* (2012) used a *Drosophila* strain that was genetically engineered to express G418-resistant genes globally or in a specific area. The response to exposure to G418, an antibiotic that blocks translation, included elevated expression of some genes, expansion of spatial expression patterns, and delays in development (STERN *et al.* 2012). These phenotypes were inherited in subsequent generations, although the descendants were not exposed to G418 (STERN *et al.* 2012). Thus, toxins can induce long lasting changes to gene expression over generations.

Olfactory experience and stress

The memory of a certain odor during early development can last long into adulthood. Using *C. elegans*, a roundworm with a simple nervous system, Remy and Hobert showed that adult animals exhibit strong responses to chemical attractants to which they were exposed during early larval development (REMY and HOBERT 2005). F1 offspring of parents who were exposed to olfactory cues also exhibited an enhanced attraction to the cues.

Mammals with more neurons and more complex neural circuitry than *C. elegans* also transmit olfactory memory to future generations. In a study led by Dias and Ressler, exposure to an odor (acetonphenone) was paired with a burst of noise that induced a startle response (DIAS and RESSLER 2013). F1 and F2 offspring of male P0 mice that had learned to link the odor with the noise, showed an increased startle response towards the conditioning odor, despite their naiveté towards the trigger (DIAS and RESSLER 2013). In addition to behavioral changes, neuroanatomical characteristics of the conditioned P0 mice were also inherited in the F1 and F2 generations (DIAS and RESSLER 2013). F1 progeny conceived by *in vitro* fertilization behaved like other animals conceived by mating, indicating that transmission of the memory of the odor-noise link occurred via gametes (DIAS and RESSLER 2013).

Postnatal exposure to stress and separation from mothers are strongly linked to anxiety and depression in adulthood (reviewed in (WEISS *et al.* 2011)), and this traumatic stress in early life can influence behaviors in future generations. Grandchildren (F3) of F1 mice who were raised under chronic stress from maternal separation exhibited depression-like behaviors, although they had not suffered from the same poor maternal care (FRANKLIN *et al.* 2010).

The role of small noncoding RNA pathway in non-genetic inheritance of *C. elegans*

RNAi-mediated transcriptional repression and RNAi inheritance in C. elegans

RNA interference (RNAi) is a gene silencing process triggered by double-stranded RNAs (dsRNAs). dsRNAs that are either endogenously expressed or experimentally introduced are cleaved to generate 21- to 25- nucleotide small interfering RNAs (siRNAs)

(ELBASHIR *et al.* 2001; BERNSTEIN *et al.* 2001). These primary siRNAs bind to the target transcripts and inhibit the translation (SEN 2006; VALENCIA-SANCHEZ 2006). Primary siRNAs also induce the amplification mechanism, where RNA-dependent RNA polymerase synthesizes de novo anti-sense mRNA by using the target mRNA as a template (SIJEN *et al.* 2001, 2007). More abundant than primary siRNAs (PAK and FIRE 2007), secondary siRNAs are crucial for effective RNAi.

Recently, studies have discovered that RNAi can also induce gene silencing in the nucleus (GUANG *et al.* 2008, 2010; FISCHER 2010). In *C. elegans*, secondary siRNAs are incorporated into a nuclear argonaute NRDE-3, which localizes to the nucleus and recruits a conserved nuclear protein NRDE-2 to block transcription of the targeted gene (GUANG *et al.* 2010). NRDE-3 is essential for RNAi targeting only a subset of genes (GUANG *et al.* 2008), indicating that transcriptional silencing is not universally required for silencing induced by RNAi. Recently, NRDE-3-dependent silencing gained a lot of attention due to its role in inheritance of silenced state triggered by RNAi. Transient exposure to dsRNA in parental generation (P0) promotes gene silencing in the subsequent generation (F1) and NRDE-3 acts in F1 to retain the memory of silenced state (Fig. 1.1) (BURTON *et al.* 2011; BUCKLEY *et al.* 2012; GU *et al.* 2012).

Locus-specific enrichment of H3K9me3, a histone modification associated with inactive transcription induced by RNAi, is involved in inheritance of RNAi-mediated silencing in *C. elegans.* When P0 animals are exposed to dsRNA, their offspring (F1) and grand-offspring (F2) exhibit H3K9me3 enrichment at the targeted locus and RNAi phenotypes, even though the trigger dsRNAs are not presented to the F1 and F2

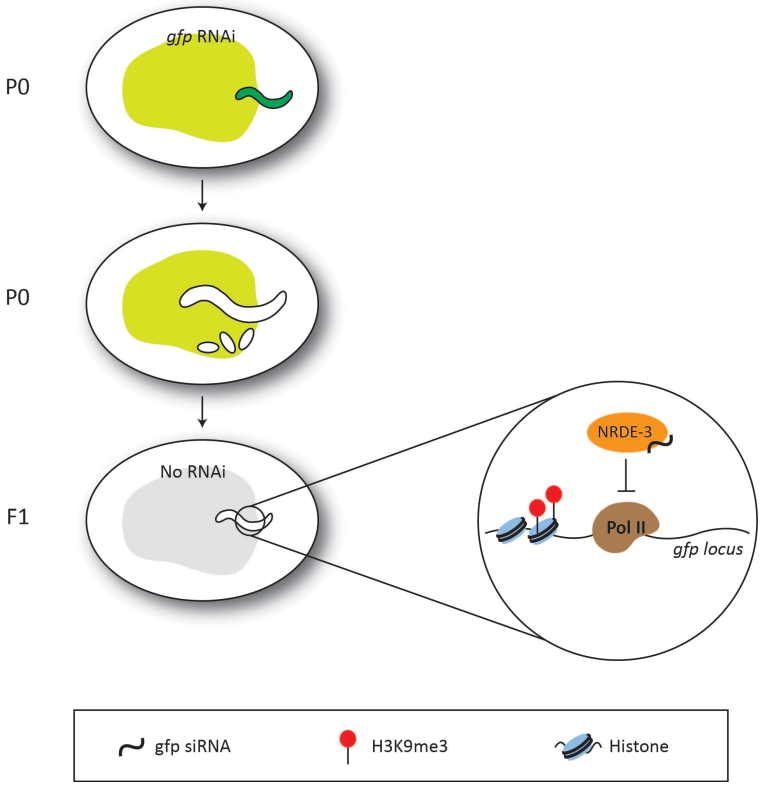


Figure 1.1. RNAi-induced silencing response is inherited to the progeny.

For example, when a transgenic animal expressing GFP (green worm; P0) is fed with GFP dsRNA-expressing bacteria, GFP expression is inhibited not only in P0 and but also in its progeny (F1) (white worms). A nuclear argonaute NRDE-3 associated with small interfering RNAs (siRNA) act in zygotes (F1) to maintain RNAi-induced silencing by blocking transcription (GUANG *et al.* 2008). In addition to siRNAs, H3K9me3 modification is enriched at the locus targeted by the initial dsRNA (GUANG *et al.* 2010; GU *et al.* 2012).

(GU *et al.* 2012). Moreover, H3K9 methyltransferases SET-25 and SET-32 are known to be required for heritable RNAi (ASHE *et al.* 2012). Does H3K9me3 act as a physical signal transmitting the memory of RNAi-induced silenced state across generations? It appears not. Temporal analysis of siRNAs and H3K9me3 in the progeny (F1) of RNAi-treated worms (P0) showed that H3K9me3 appeared at later developmental stages compared to siRNAs, indicating that H3K9me3 was re-established in the progeny, not transmitted from the previous generation (BURTON *et al.* 2011).

Other small RNA pathways in C. elegans that are involved in the transmission of gene expression states across generations

In addition to the exogenous RNAi pathway, *C. elegans* employs other small RNA pathways to maintain transcriptional states across generations In the *C. elegans* germ line, transgenes tend to be silenced robustly and this is mediated by piRNAs, one of the major classes of small RNAs. Unlike the aforementioned primary and secondary siRNAs, piRNAs are genetically encoded and largely, but not exclusively, expressed in the germ line (LIN 2007). PRG-1, a *C. elegans* Piwi protein involved in the biogenesis of piRNAs (BAGIJN *et al.* 2012; LEE *et al.* 2012), is required to trigger silencing of a transgene in the germ line (ASHE *et al.* 2012; SHIRAYAMA *et al.* 2012). Once activated, the piRNA pathway induces the production of another class of small RNAs known as 22G-RNAs, which are loaded onto different argonautes and maintain the silenced state of the transgene over multiple generations (BAGIJN *et al.* 2012; LEE *et al.* 2012).

Not only is silencing remembered, but also gene activity. Recent studies have uncovered an “anti-silencing” pathway that involves a different cohort of 22G-RNAs

(CLAYCOMB *et al.* 2009; SETH *et al.* 2013; WEDELES *et al.* 2013). Seth *et al* (2013) and Wedeles *et al* (2013) relied on transgenes and discovered that an actively expressed transgene not only maintained its active transcriptional state, but could also de-repress a silenced transgene that shared sequence identity (SETH *et al.* 2013; WEDELES *et al.* 2013). This phenomenon (known as RNA-induced epigenetic gene activation or RNAa) is mediated by the argonaute CSR-1 (CLAYCOMB *et al.* 2009; SETH *et al.* 2013; WEDELES *et al.* 2013) (Fig. 1.2). Given that piRNAs and 22G-RNAs target endogenous genes (CLAYCOMB *et al.* 2009; BAGIJN *et al.* 2012; LEE *et al.* 2012), small RNA pathways appear to play a crucial role in transmitting the memory of endogenous vs. foreign gene expression from one generation to the other. While these pathways were discovered using transgenes, the phenotypes of argonaute mutants (e.g. *csr-1 and alg-3/alg-4*) and some RNAi-hypersensitive mutants (e.g. *eri-1* and *rrf-3*) indicate a critical role in gametogenesis (PAVELEC *et al.* 2009; CONINE *et al.* 2010, 2013).

Steroid hormone signaling pathway in *C. elegans*

My thesis reveals a new role of the *C. elegans* nuclear hormone receptor (NHR) DAF-12 in RNAi. NHR are transcription factors whose activity depends on ligands and on interacting partners; when lipophilic hormones bind to an NHR, it recruits various chromatin modeling enzymes and transcription co-activators to promote expression of downstream genes. In contrast, interactions between NHRs and their co-repressors result in repression of target genes. Humans and *Drosophila* each have fewer than 50 NHRs, whereas there are more than 284 NHRs in the genome of *C. elegans* (SLUDER and MAINA 2001; GISSENDANNER *et al.* 2004).

Figure 1.2. The role of CSR-1/Argonaute in transactivation of silenced gene.

In *C. elegans*, the many transgenes expressed in the germ line are susceptible to silencing (SHIRAYAMA *et al.* 2012). Transgenes that are active are associated with H3K4me2 (blue; generally considered as a mark of active transcription) whereas transgenes that are silenced are enriched for H3K9me3 (red; a histone modification linked to silenced loci) (WEDELES *et al.* 2013). When an animal carrying a silenced transgene (gfp1) is mated with a worm carrying actively transcribed transgene (gfp2), the resulting F1 inherits CSR-1/Argonaute from the gfp2-expressing P0 animal. In F1, CSR-1 counteracts the surveillance mechanism that silences gfp1 expression (1) (SETH *et al.* 2013; WEDELES *et al.* 2013). In addition, RNA-induced epigenetic silencing (RNAe), where the surveillance mechanism acting on a genetic locus targets another locus with similar DNA sequence (SHIRAYAMA *et al.* 2012), is inhibited by CSR-1 (2) (WEDELES *et al.* 2013)**.** The observation that *csr-1* -/- mutants lack H3K4me2 in germline (CONINE *et al.* 2013) indicates that CSR-1 activity is correlated with H3K4me2 enrichment (3). Owing to the activity of CSR-1 bestowed by the animal expressing gfp2, the silenced state of gfp1 is reversed to the active transcription state and gfp2 is protected from silencing mechanism in F1. Even when gfp2 is removed in F2, the memory of gfp1 transcription is inherited, thus gfp1 remains to be expressed. Although it remains to be seen, we expect that accumulation of H3K4me2 would accompany the reversal of a silenced transgene to active transcription.

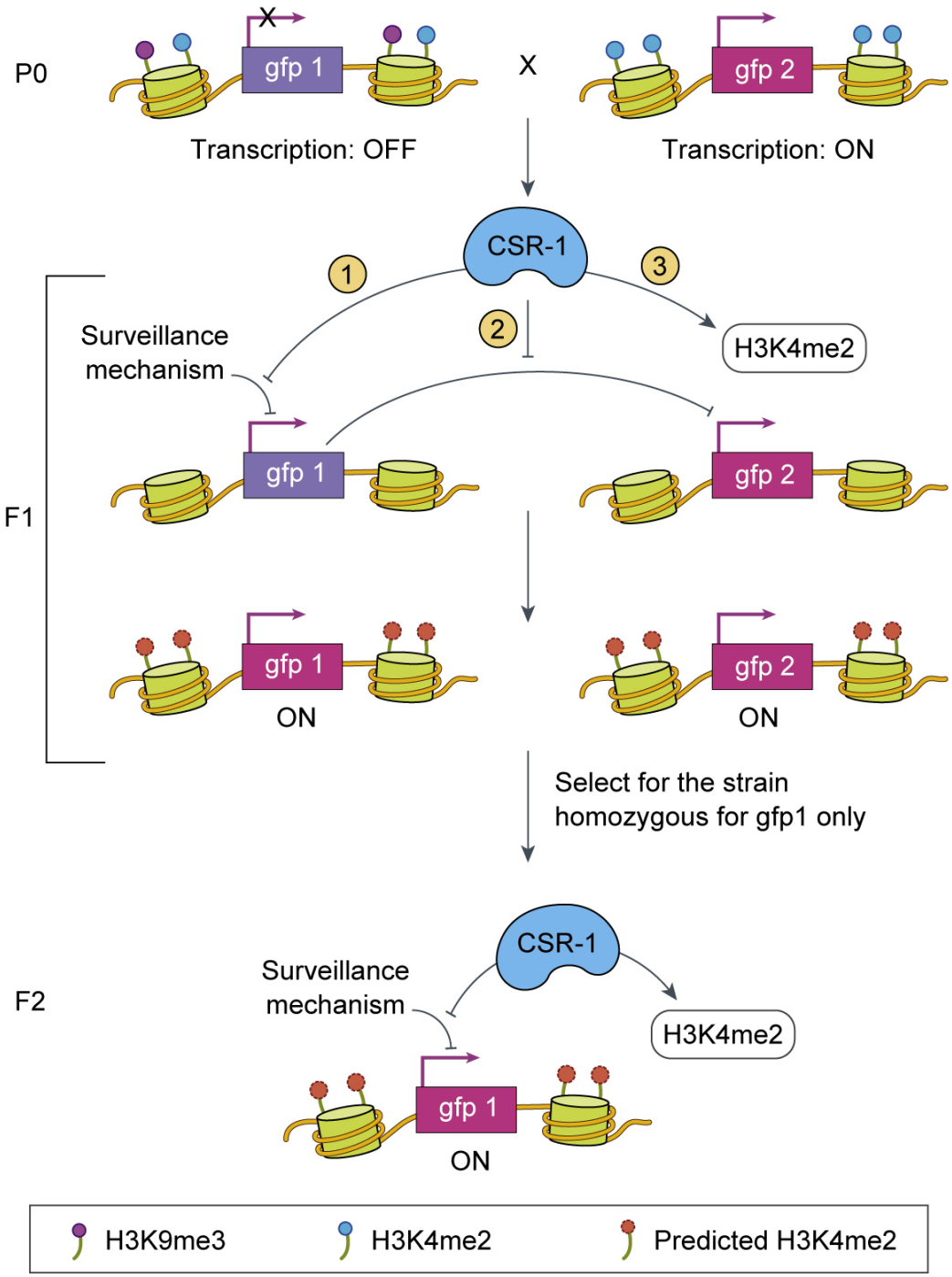


Figure 1.2 (continued). The role of CSR-1/Argonaute in transactivation of silenced gene.

As a nuclear hormone receptor, DAF-12 binds to the promoter regions of target genes and, depending on its binding partner, DAF-12 acts as an activator or a repressor (AO *et al.* 2004; SHOSTAK *et al.* 2004; FISHER and LITHGOW 2006; HOCHBAUM *et al.* 2011). One of the unique characteristics of DAF-12 is its link to the environmental conditions. Pathways that respond to environmental cues converge at DAF-12 (reviewed in (FIELENBACH and ANTEBI 2008)), indicating its role in mediating physiological changes in response to environmental conditions. Here, I discuss the regulation of the DAF-12 activity and its roles in *C. elegans* development.

Ligands

The level of Dafachronic acid (DA; 3-keto-sterols) determines the function of DAF-12. Dafachronic acids are the first steroid hormone discovered as a ligand of NHR in nematodes (MOTOLA *et al.* 2006; MAHANTI *et al.* 2014). Their precursors are dietary cholesterol, which is metabolized through successive oxidation by multiple enzymes. The enzyme network required for DA synthesis from cholesterol is rather complex and divergent. Enzymes are expressed in different tissues, including the intestine, the XXX neuroendocrine cells and the hypodermis (OHKURA *et al.* 2003; ROTTIERS *et al.* 2006; SCHAEDEL *et al.* 2012). A small amount of DA produced in the XXX neuroendocrine cells located in the head increases the production of DA in the hypodermis, and this amplification allows animals to make a binary and irreversible decision—reproductive growth or dauer entry—in response to a small change in the DA synthesis (SCHAEDEL *et al.* 2012).

In the presence of its ligands, NHRs interact with co-activators, which facilitate the transcriptional activity of NHRs. When expressed in mammalian cells, DAF-12 binds to steroid receptor coactivator 1, but its endogenous coactivator has not bee identified yet (MOTOLA *et al.* 2006; MAHANTI *et al.* 2014).

Co-repressor DIN-1

Another important domain in DAF-12 is a site where its co-repressor DIN-1S binds. DIN-1S binding site is embedded in the LBD, and the presence of DA disassociates DIN-1S from DAF-12 (MOTOLA *et al.* 2006). Repressor activity of DIN-1S was first inferred from its similarity to human SMRT/HDAC-1-associated repressor protein (SHARP). Mammalian studies have shown that through its highly conserved C-terminal domain, SHARP interacts with transcriptional repressors, such as SMRT and nuclear receptor co-repressors (NCoR) and consequently recruits histone deacetylase (HDAC) (SHI 2001; MIKAMI *et al.* 2014). SHARP binds to unliganded nuclear hormone receptors and represses their transcriptional activity (SHI 2001; SHI *et al.* 2002). Yeast-two hybrid experiments have shown that DIN-1S binds to DAF-12, and the mutant analysis revealed that the dauer phenotype of *daf-12* mutants was dependent on DIN-1S. Together, previous works strongly suggest that DIN-1S is a co-repressor for DAF-12 (LUDEWIG *et al.* 2004).

Expression pattern of DAF-12

Two independent studies using either GFP fused to the *daf-12* promoter or Northern blot analysis reported that *daf-12* is expressed from mid-embryogenesis to adulthood (ANTEBI

et al. 2000; SNOW and LARSEN 2000). The long and short isoforms of *daf-12* have similar temporal expression patterns (SNOW and LARSEN 2000). *daf-12* is expressed in many tissues including neurons, intestine, pharynx and somatic gonad (ANTEBI *et al.* 2000). Studies that profiled the transcriptome in the germ line and early embryos did not detect *daf-12* mRNA (BAUGH *et al.* 2003; REINKE *et al.* 2003; YUZYUK *et al.* 2009; LEVIN *et al.* 2012). These results indicate that no or few *daf-12* transcripts are maternally loaded into oocytes and able to function in early embryos.

Biological roles of DAF-12

As a part of the endocrine system in *C. elegans*, DAF-12 plays a crucial role in the processes that require systemic changes, such as larval development, aging, and immune responses. DAF-12 binds to the promoter regions of target genes and, depending on its binding partner, DAF-12 acts as an activator or a repressor (AO *et al.* 2004; SHOSTAK *et al.* 2004; FISHER and LITHGOW 2006; HOCHBAUM *et al.* 2011). It is important to note here that studies up to date mainly focused on the role of DAF-12 during the development of one generation. My thesis reveals that DAF-12 is also involved in cross-generational signaling. .

Dauer entry

daf-12 was first discovered from the EMS mutagenesis screening designed to identify mutants defective in the dauer larval formation (daf) (RIDDLE *et al.* 1981). Once hatched, *C. elegans* normally go through four larval stages and become reproductive adults. However, if animals encounter an unfavorable environment, such as overcrowding,

scarce food and/or high temperature, they choose an alternative third larval stage called the dauer (FIELENBACH and ANTEBI 2008). Dauers remodel their cuticle (CASSADA and RUSSELL 1975), suppress pharyngeal pumping (CASSADA and RUSSELL 1975), and oxidize stored fat to produce energy (WADSWORTH and RIDDLE 1989). These physiological and metabolic changes allow dauers to tolerate harsh environmental conditions better than developing larvae and can survive for months (CASSADA and RUSSELL 1975). Once the environment becomes favorable, dauers resume reproductive growth.

The decision whether to grow to adulthood or form a dauer is an excellent example of how environmental inputs can influence the development of an organism, and DAF-12 acts at a convergence point for different environmental sensing pathways. Favorable environmental cues trigger signaling pathways such as insulin and TGF-beta pathways (REN *et al.* 1996; KIMURA *et al.* 1997). Activation of such pathways leads to production of DA, which binds to DAF-12 and promotes larval development (MOTOLA *et al.* 2006; GERISCH *et al.* 2007; SCHAEDEL *et al.* 2012; MAHANTI *et al.* 2014). In contrast, unfavorable environments block the biosynthesis of DA (REN *et al.* 1996; SCHACKWITZ *et al.* 1996; GERISCH *et al.* 2001), and as a result, a repressor complex of DAF-12 promotes dauer formation (LUDEWIG *et al.* 2004).

There are more than twenty *daf-12* mutations identified so far and their dauer phenotypes largely depend on the nature of the molecular lesions. In general, animals that bear mutations in the DNA binding domain cannot form dauer (Daf-d) when challenged by unfavorable environmental conditions (ANTEBI *et al.* 2000). This result indicates that DNA binding and presumably transcriptional control is important for dauer entry.

Molecular lesions in the LBD lead to either Daf-d or Daf-c (constitutive entry into dauer) (ANTEBI *et al.* 2000), suggesting that a subtle change in the ligand binding domain alters the affinity for DA or DIN-1 and consequently affects the dauer phenotype.

The role of DAF-12 in mediating the environmental effect on physiological changes is conserved in nematodes. The nematode *Pristionchus pacificus* forms teeth-like denticles when they are starved or presented to a high level of pheromone, an indicative of high population density (KIONTKE and FITCH 2010). Bento et al (2010) found that the formation of a wide stoma with teeth induced by starvation depends on DAF-12 function and DA level (BENTO *et al.* 2010). Taken together, DAF-12 is an integral part of the mechanism that translates the environmental signals into physiological changes.

Physiological capacitor

The role of DAF-12 for dauer entry shows that DAF-12 executes important decisions in development. Recently, Hochbaum, Fisher and colleagues reported another example showing the executive role of DAF-12. They reared wildtype worms and *daf-12* (rh61rh411; null) mutants while cycling between 15°C (4 hours) and 25°C (4 hours) until adulthood (HOCHBAUM *et al.* 2011). The majority of wildtype worms had the same number (16) of hypodermal seam cells as wildtype animals that grew at 20°C, whereas a significant portion of *daf-12* null mutants that were challenged with temperature fluctuations underwent an extra hypodermal seam cell division (HOCHBAUM *et al.* 2011). This observation indicates that DAF-12 buffers perturbations in the surroundings, and thus lack of DAF-12 leads to hypersensitivity to even small environmental changes.

Developmental timing

In addition to dauer phenotypes, another prominent trait associated with some, but not all, *daf-12* mutations is defects in seam cell division and in gonad migration, which normally occur at specific larval stages (ANTEBI *et al.* 1998, 2000). Two independent studies have shown that DA-bound DAF-12 promotes the transition from the second larval stage (L2) to the third larval stage (L3) by activating *let-7* family microRNAs (BETHKE *et al.* 2009; HAMMELL *et al.* 2009), which down-regulate *hbl-1* (hunchback), an essential transcription factor for the L2 stage (ABBOTT *et al.* 2005). In addition to the *let-7* family of microRNAs, many heterochronic genes, which encode factors essential for developmental timing mechanisms, have a DAF-12 binding site in their promoters and their expression is altered in *daf-12* null mutants (HOCHBAUM *et al.* 2011). Together these studies imply that DAF-12 regulates heterochronic pathways.

Aging

DAF-12 has drawn a lot of attention recently for its role in inter-organ communication involved in aging, particularly in the gonad. When two germline precursor cells are ablated during early larval development, *C. elegans* live longer than germline-intact wildtype animals by 60% (HSIN and KENYON 1999; ARANTES-OLIVEIRA *et al.* 2002). Germline-ablated worms require DAF-12 and DAF-9, a cytochrome P450 essential for DA synthesis, to extend their lifespan (HSIN and KENYON 1999; GERISCH *et al.* 2001; MOTOLA *et al.* 2006; ROTTIERS *et al.* 2006; YAMAWAKI *et al.* 2010). This discovery suggests that DAF-12 and DA act as "anti-aging" factors that promote the longevity of germline-deficient animals. How could the DAF-12 pathway prevent aging in response to

germline ablation? A recent report discovered that germline ablation elevates the level of DA at the fourth larval and young adult stages (SHEN *et al.* 2012). DA-bound DAF-12 stimulates the expression of two key microRNAs*, mir-84* and *mir-241* (SHEN *et al.* 2012). These microRNAs down-regulate their target genes, thus activating DAF-16/FOXO signaling (SHEN *et al.* 2012), a major pathway eliciting the anti-aging effect in germline-less animals (HSIN and KENYON 1999; LIN *et al.* 2001; BERMAN and KENYON 2006). In addition, DAF-12 activates downstream genes that are distinct from those regulated by DAF-16/FOXO, to extend the lifespan of germline-ablated animals (YAMAWAKI *et al.* 2010). Taken together, the DA and DAF-12 signaling pathway mediates a systemic effect in longevity in *C. elegans*.

Another example of DAF-12 inducing systemic physiological changes is relevant to mating-triggered shrinking. Shi and Murphy (2013) observed that when hermaphrodites were mated with males, they shrank massively and their post-reproductive longevity was shortened to > 40% of that of unmated hermaphrodites (SHI and MURPHY 2013). *daf-9* and *daf-12* null mutants do not show such phenotypes after mating, indicating their roles in mating-triggered changes (SHI and MURPHY 2013). Mating decreased the expression of *daf-9* in the hermaphrodite' spermatheca, a part of the somatic gonad that stores sperm (SHI and MURPHY 2013). Given that DAF-12 regulates the expression of the genes required for osmotic stress resistance (FISHER and LITHGOW 2006; MCCORMICK *et al.* 2012), the authors speculated that mating decreases the level of liganded DAF-12 in hermaphrodites, which in turn causes post-mating shrinking (SHI and MURPHY 2013). The causal link between shrinking and the immediate death after reproduction has not been tested yet. However, this study suggests that as an integral part

of endocrine system in *C. elegans*, DAF-12 sets off systemic responses to a local signaling cue.

To summarize, my thesis uncovers a new role for DAF-12 in cross-generational signaling that regulates the robustness of RNAi. Previous works highlighting the importance of DAF-12 in translating environmental inputs into molecular and physiological changes also led us to test and found an interesting link between parental experience of a certain environment and RNAi efficiency in offspring.

CHAPTER II

The Role of DAF-12/NHR in RNAi regulation via Cross-generational Signaling

Introduction

Haploid germ cells carry parental genetic information that will constitute the genetic makeup of a zygote. In addition to DNA, germ cells have organelles and lots of nuclear and cytoplasmic molecules that significantly influence their offspring's development, behavior and health. The classic example is maternal-effect genes, which are expressed and packaged as RNA or protein in oocytes, and which play a crucial role in early events of embryogenesis (reviewed in (LI *et al.* 2010)). Another example is mother-to-offspring immune priming in vertebrates, where mothers transmit antibodies to their offspring to improve their chances of survival (MOUSSEAU and FOX 1998; GRINDSTAFF *et al.* 2003; HASSELQUIST and NILSSON 2009). Thanks to technologies that allow detection of miniscule amounts of biological molecules and increasing knowledge about epigenetic marks such as histone modifications and DNA methylation, a list of molecules deposited in germ cells is ever expanding. More recent studies have suggested that parental experiences in different environments can cause abnormal phenotypes in their offspring without any genetic mutations. A flurry of studies has attempted to identify non-genetic molecules responsible for this non-conventional inheritance, and strong candidates include DNA methylation, histone modifications, and small non-coding RNAs (ANWAY *et al.* 2005; JABLONKA and RAZ 2009; BURTON *et al.* 2011; GREER *et al.* 2011; BUCKLEY *et al.* 2012; GU *et al.* 2012; LIM and BRUNET 2013) .

Inheritance of gene silencing by RNA interference (RNAi) in *C. elegans* illustrates the memory of transient experiences across generations. RNAi is a cellular mechanism that silences a specific gene whose sequence is complementary to double stranded RNAs (dsRNAs). In a typical experiment, exogenously introduced long dsRNAs are first cleaved into 21- to 25-nucleotide small interfering RNAs (primary siRNAs) (ELBASHIR *et al.* 2001; BERNSTEIN *et al.* 2001), which bind to the mRNA transcripts of the endogenous target and inhibit their expression at the post-transcriptional level (SEN 2006; VALENCIA-SANCHEZ 2006). Effective RNAi relies on the second class of siRNAs (secondary siRNAs), de novo RNA molecules that are synthesized using the targeted transcripts as a template and produced by the activity of RNA-dependent RNA polymerase (SIJEN *et al.* 2001, 2007). Secondary RNAis triggers transcriptional gene silencing via the nuclear RNAi defective (NRDE) pathway (GUANG *et al.* 2008). Interestingly, exposure of worms (P0) to double-stranded RNA can induce RNAi not only in P0 animals, but also across multiple generations (F1, F2, etc) (FIRE *et al.* 1998; GRISHOK *et al.* 2000; VASTENHOUW *et al.* 2006; ALCAZAR *et al.* 2008). The identity of heritable molecules responsible for RNAi inheritance is not known yet. However, persistence of RNAi is associated with the presence of siRNAs and enrichment of H3K9me3 at the locus targeted by the initial trigger dsRNA (BURTON *et al.* 2011; BUCKLEY *et al.* 2012; GU *et al.* 2012).

Here, we show that DAF-12, a *C. elegans* nuclear hormone receptor, modulates cross-generational RNAi. DAF-12 has well described roles in the response to environmental conditions, developmental timing and entry into dauer, a developmental

arrest induced by harsh conditions (ANTEBI *et al.* 2000; FIELENBACH and ANTEBI 2008; BETHKE *et al.* 2009; HAMMELL *et al.* 2009). Transcriptional activation and repression by DAF-12 depend on its ligand Dafachronic acid (DA)—a metabolite of dietary cholesterol—and on the DAF-12 co-repressor DIN-1, respectively (LUDEWIG *et al.* 2004; GILL *et al.* 2004; MOTOLA *et al.* 2006; HELD *et al.* 2006). Whereas previous studies on DAF-12 focused on its role in the life history of one generation, we find that DAF-12 in mothers promotes transcriptional repression induced by RNAi in her progeny. The function of *daf-12* in RNAi is independent of its roles in developmental timing and dauer entry. We also found that the environmental conditions of the mothers' larval development determines the effect of DAF-12 on RNAi efficiency in zygotes. Together, these results demonstrate that maternal DAF-12 not only creates additional layers of regulation in RNAi inheritance, but also buffers the environmental contribution to RNAi.

Materials and Methods

Strains and maintenance

Worms were maintained as described before (BRENNER 1974). SM2020 *daf-12* (rh61rh411) strain used for most of the experiments in this study was outcrossed six times and paired with its wildtype sibling (SM2021) for all the experiments. The complete list of *C. elegans* strains used in this study is in Appendix III. Worms were reared at 20°C except *pha-4*(ts) and *daf-7*(e1372), which were maintained at 24°C and 15°C, respectively.

RNA interference (RNAi)

Bacteria expressing double-stranded RNA were obtained from the Ahringer library (KAMATH and AHRINGER 2003) and sequenced to confirm their identities. *pha-4* RNAi clones made previously by the Mango lab (bSEM 865) (KIEFER *et al.* 2007) were used for most *pha-4* RNAi assays except for Pol II ChIP and RT-qPCR experiments, where *pha-4* RNAi clones from the Ahringer library were used. There are two differences between bSEM865 and the Ahringer library *pha-4* RNAi clones. First, bSEM865 produces *pha-4* dsRNA that is as long as the complete *pha-4* transcript, whereas the Ahringer library clone generates dsRNA that corresponds to exon 5, intron 5, and exon 6 of the *pha-4* sequence (F38A6.1a). Second, the backbone vector for bSEM865 is pPR244 (REDDIEN *et al.* 2005), while the Ahringer library clone used the L4440 feeding vector (KAMATH and AHRINGER 2003).

RNAi clones were cultured in 5ml LB with antibiotics (final concentration of 50μg/ml) in 13ml round-bottom tube for 8 hours at 37°C. LB was added to the culture to

achieve the optical density (OD) of 1.5. To vary the strength of RNAi, empty vector (L4440) or mCherry RNAi clones (bSEM1098; full mCherry sequence is inserted into pPR244) were added to the RNAi culture. The mixture was spun down at 2500rpm for 10min and resuspended in 8mM IPTG solution (Gold Biotechnology #12481C100) with 60μg/ml Carbenicillin (Gold Biotechnology #C-103-25) to achieve the final OD of 15. For *pha-4* RNAi (bSEM 865), bacteria were resuspended with M9 only. 100ul of the bacterial mixture was spotted on unseeded NGM small plates (diameter of 35mm). They were incubated at 20°C for two days.

For RNAi assays, 10 L4 worms (P0) were placed on a medium OP50-seeded plate and cultured at 25C for 2.5 days. Their progeny (F1) at the L4 stage were first moved to an empty vector plate to minimize the amount of OP50 bacteria transferred to the RNAi plates. After allowing worms to crawl to get rid of OP50 bacteria on their cuticle, 15 F1 L4 worms were placed on a RNAi plate and cultured at 25°C for 22-24hr. The embryos (F2) were moved to an OP50 seeded plate and reared at 25°C. Two days later, the number of worms that show wildtype phenotype (i.e. survival or fertility) was recorded.

Immunohistochemistry

Embryos were collected from plates, washed with M9 three times, and placed on a poly-lysine coated slide. For PHA-4 antibody staining, embryos were fixed with 2% paraformaldehyde solution under a coverslip. The slides were incubated in a humidity chamber for 25 min and then placed on a metal plate on dry ice for at least 10 min. For DLG-1 staining, embryos were covered with a coverslip and directly placed on the metal plate on dry ice for more than 30 min. The coverslip was popped off to crack and

permeabilize embryos. Then, the slides were submersed in ice-cold methanol for 3 min for PHA-4 staining or 5 min for DLG-1 staining and washed twice with 1x TBST (Tris-Buffered Saline Tween) for 5 min at room temperature. To prevent non-specific staining, the embryos were incubated with 50ul of TNB (0.1 M Tris–HCl, 0.15 M NaCl, 0.05% Tween 20, pH 7.5 containing blocking reagent (NEN)) containing 10% NGS (Normal goat serum; Jackson ImmunoResearch #113435) in a humidity chamber at room temperature for 30 min. The PHA-4 primary antibody (KALTENBACH *et al.* 2005) and/or DLG-1 antibody (Thermo #NG159845) were added to the slides, which then were placed in a humidity chamber and incubated at 15°C. After washed with 1x TBST three times, the slides were incubated with the secondary antibodies (Invitrogen) for two hours in a humidity chamber at room temperature. 7ul of DAPI solution (Invitrogen #S36938) was added to the slide and the coverslip was placed. The edge of the coverslip was sealed with a nail polisher.

Microscopy and image processing

Images were obtained using Zeiss LSM780 confocal microscope (a 63x PlanApo oil immersion lens with 2x digital zoom). Each z-stack image was 0.32μm apart. Using Image J, a maximum intensity projection image was obtained per embryo. Then, the intensity of signals in the pharyngeal cells was measured by using Volocity Image Analysis Software (PerkinElmer).

***pha-4*(ts) strains**

pha-4(ts) strains have a *pha-4* nonsense mutant allele, either q500 or zu225, in *smg-1*(cc546ts) background. *pha-4*(q500) and *pha-4*(zu225) bear nonsense mutations in aa349 and aa384, respectively (KALTENBACH *et al.* 2005). Normally, *pha-4*(q500) and *pha-4*(zu225) transcripts are rapidly degraded by the nonsense-mediated decay system, a process responsible for eliminating transcripts bearing nonsense mutations, and most of homozygous mutants show embryonic lethality (KALTENBACH *et al.* 2005). However, when the nonsense-mediated decay process is compromised, truncated but functional PHA-4 proteins are produced and mutants become viable (KALTENBACH *et al.* 2005). By having temperature sensitive allele of *smg-1*, which encodes an essential component of the NMD pathway, in the background of *pha-4*(q500) or *pha-4*(zu225) mutants, we can control the accumulation of truncated PHA-4 proteins (KALTENBACH *et al.* 2005). For instance, the NMD pathway is inactive at 24°C, thus *smg-1*(cc546ts); *pha-4*(zu225) animals could grow normally, whereas they show severe defects in pharynx development when reared at 15°C, at which the NMD pathway is rigorous and targets *pha-4*(zu225) transcripts for degradation (KALTENBACH *et al.* 2005; UPDIKE and MANGO 2007). At the intermediate temperature of 20°C, *smg-1*(cc546ts); *pha-4*(zu225) animals show some PHA-4 protein accumulation, but they fail to pass the first larval stage (KALTENBACH *et al.* 2005; UPDIKE and MANGO 2007).

Chromatin immunoprecipitation (ChIP)

Worms were fed with empty vector RNAi or *pha-4* RNAi as described above. Approximately, 150,000 embryos were frozen in liquid nitrogen and thawed in ice. They

were fixed with 1.5% formaldehyde solution for 30 min at room temperature. Then, glycine (final concentration of 0.125M) was added to quench the formaldehyde. After 15 min of incubation, embryos were carefully washed with pre-chilled M9 containing 1x protease three times. Fixed embryos were resuspended in nuclear lysis buffer (50mM Tris pH 8.0, 10 mM EDTA pH 8.0, 0.2 % SDS) containing 1x PhosStop (Roche #04906845001) and 1x protease. The samples were sonicated using QSonica Q700 Sonicator with a program containing 20 cycles of 20 sec on, 20 sec off. Two steps preceded immunoprecipitation. First, fragmented chromatin was cleaned by incubating with 25µl of protein A agrose beads in 1ml of ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl pH 8.1, 167mM NaCl) for an hour. Second, 2µg of RNA polymerase II antibody (CTD4H8; Covance #MMS-128P) or 4µg of H3K9me3 antibody (Abcam #ab8898) was incubated with 25µl of protein G magnetic beads for at least 6 hours at 4°C. 45-50µg of sheared chromatin was incubated overnight with magnetic beads-bound RNA polymerase II antibody at 4°C. 10% of sonicated chromatin was also saved as an input at -20°C overnight. After incubation of antibody and chromatin, the protein/DNA complex was incubated with 1ml of the following buffers twice for 5 min on the rotator to remove any chromatin that was unbound to the antibody; ChIP dilution buffer, low salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH 8.1, 150mM NaCl), high salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH 8.1, 500mM NaCl), and LiCl buffer (EMD Millipore #20-156). Chromatin was lastly washed with TE buffer for three times before proceeding to the next step. To reverse cross-links, the antibody-chromatin mixture as well as input chromatin were incubated with 150µl of ChIP elution buffer (1% SDS,

50mM NaHCO3, 140mM NaCl) at 55°C for an hour. Then, RNAse A (0.06mg/ml) was added to each tube and incubated at 37°C for 30 min to degrade RNAs. Then, the samples were incubated with proteinase K (0.26μg/μl) at 65°C overnight. DNA recovered from ChIP samples and input DNA were collected by using QIAquick PCR purification kit (QIAGEN #28106). Purified DNA was run on a 2% agrose gel, and 100-600 bp fragments were isolated for further qPCR analysis.

qPCR and analysis

Quantitative PCR was performed following the instruction provided by KAPA SYBR FAST Universal qPCR kit (KAPA Biosystem #KK4602). For qPCR following Pol II ChIP, 0.01μg to 10μg genomic DNA were used to construct a standard curve. When cDNA was used as a template for qPCR, the expression level of F35G12.2 was used as a reference. At least one of the qPCR primer pair was designed to span an exon-exon junction to avoid amplification of genomic DNA. The sequences of the primers used in this study are in Appendix IV. To determine the difference in gene expression between wildtype and *daf-12* mutants, the "delta-delta Ct method" (LIVAK and SCHMITTGEN 2001) was used.

RNA extraction

Young adult worms were washed off from plates and treated with the bleach solution (0.75% bleach containing 0.25M NaOH) to obtain early embryos. The embryos were snap-frozen in liquid nitrogen. To the partially thawed embryos, the same volume of glass beads (Sigma #G8772) and 1ml of Trizol were added. After 30 sec of vortex

followed by 3min of incubation at room temperature, 300µl of chloroform was added to the worm mixture, which was then shaken for 15 sec and incubated at room temperature for 3 min. The mixture was centrifuged at 14,000rpm for 15 min at 4°C, and the aqueous phase was transferred to a new tube. An equal volume of cold (4°C) isopropanol, 1/10 volume of 3M sodium acetate (pH 5.5) (Calbiochem #567422), and 1ul of GlycoBlue (Ambion #AM9516) were added to the aqueous phase and incubated at -20°C for longer than 30 min. After spinning down the tubes at 14,000 rpm for 15min at 4°C, the supernatant was removed and the blue pallet was washed with 70% cold (-20°C) ethanol. The tubes were centrifuged again at 9500rpm for 5min at 4°C, and the supernatant was removed. The pellet was air-dried for about 5 min and dissolved in 50ul of Nuclease-free water. RNA purification was performed using RNA Clean & Concentrator kit (Zymo Research #R1015) following the protocol provided by the manufacturer. 110ng - 1ug of total RNA was used to synthesize cDNAs (ProtoScript First Strand cDNA Synthesis kit, New England BioLabs #E6300S). To avoid DNA-RNA duplex formation, which can impede quantitative PCR analysis, the resulting cDNAs were incubated with RNAse H (New England BioLabs #M0297S) at 37° for 30 min followed by incubation at 65°C for 20 min to inactivate the enzyme.

Results

DAF-12 is required for robust pha-4 RNAi

We chose to use *pha-4* RNAi to test the role of DAF-12 in RNAi. PHA-4 is the *C. elegans* orthologue of the FoxA transcription factor required for the development of pharynx (or foregut), the feeding organ that pumps the food into the intestine for digestion (HORNER *et al.* 1998; GAUDET and MANGO 2002; MANGO 2009). Low expression of *pha-4* in embryos causes defects in pharynx formation, thus leading to larval lethality (MANGO *et al.* 1994). Such conspicuous *pha-4* RNAi phenotypes—death soon after hatching—allowed us to track changes in the *pha-4* RNAi efficiency.

To sensitize the RNAi assay, we diluted *pha-4* dsRNA-expressing bacteria with different amounts of neutral bacteria (Fig. 2.1). This assay allowed us to detect any subtle differences between wildtype and *daf-12*(rh61rh411; null) mutants in the severity of the *pha-4* RNAi phenotype, which could be otherwise masked when a high dose of dsRNA is provided (TIMMONS *et al.* 2003; HABIG *et al.* 2008; ZHUANG and HUNTER 2011). When wildtype and *daf-12* mutants were exposed to a range of dsRNA targeting *pha-4*, we found that *daf-12* mutants had better viability than wildtype animals, typically two to three fold (Fig. 2.2).

As another way of determining a low RNAi efficiency in *daf-12* mutants, we examined PHA-4 protein in embryos after strong *pha-4* RNAi. Consistent with the weak *pha-4*(RNAi)-associated lethality in *daf-12* mutants, PHA-4 accumulation was two fold higher in *daf-12* mutants than in wildtype embryos (Fig. 2.3). This observation supports the conclusion that DAF-12 is required for robust *pha-4* RNAi, and implies that DAF-12 modulation occurs prior to or during mid-embryogenesis.

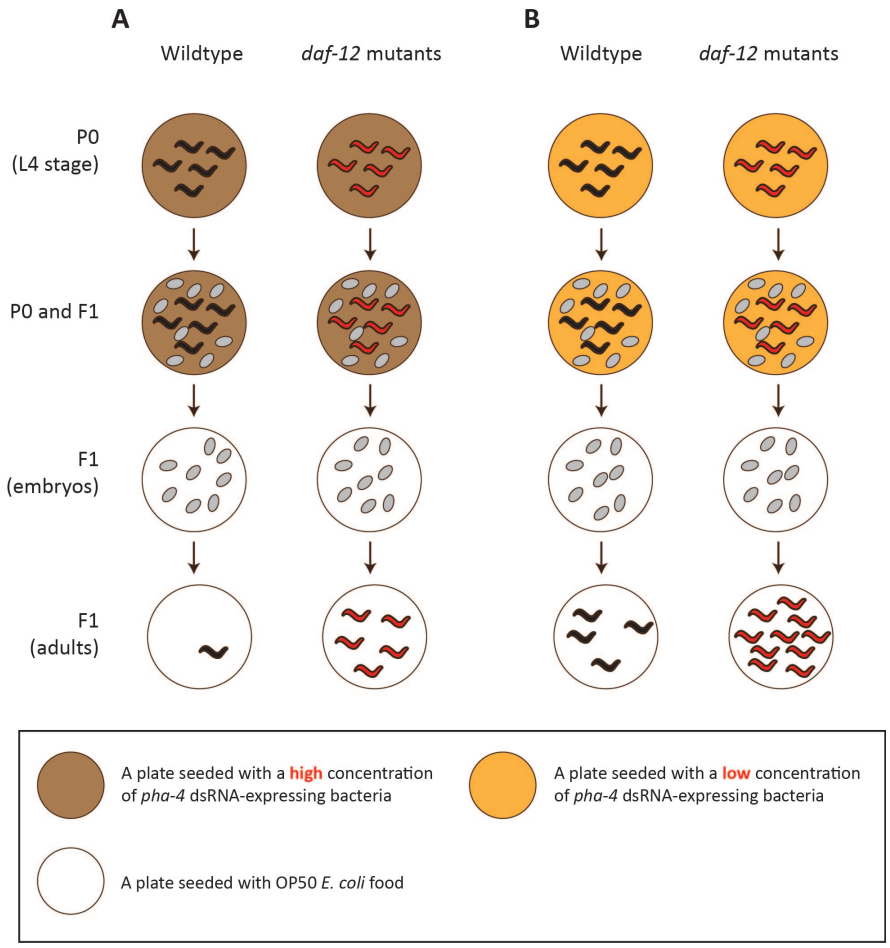


Figure 2.1. A schematic diagram of the *pha-4* RNAi assay used for this study.

10-15 wildtype (black) and *daf-12* mutants (red) (P0) at the fourth larval stage (L4) were fed for 24hr with *pha-4* dsRNA-expressing bacteria mixed with control (empty vector) RNAi bacteria. We made various dilutions of *pha-4* dsRNA-expressing bacteria (less diluted, thus strong *pha-4* RNAi (A); highly diluted, thus weak *pha-4* RNAi (B)) to sensitize the assay. Embryos (F1) were moved to the plates seeded with normal OP50 *E.coli* food and the viability of F1 was scored.

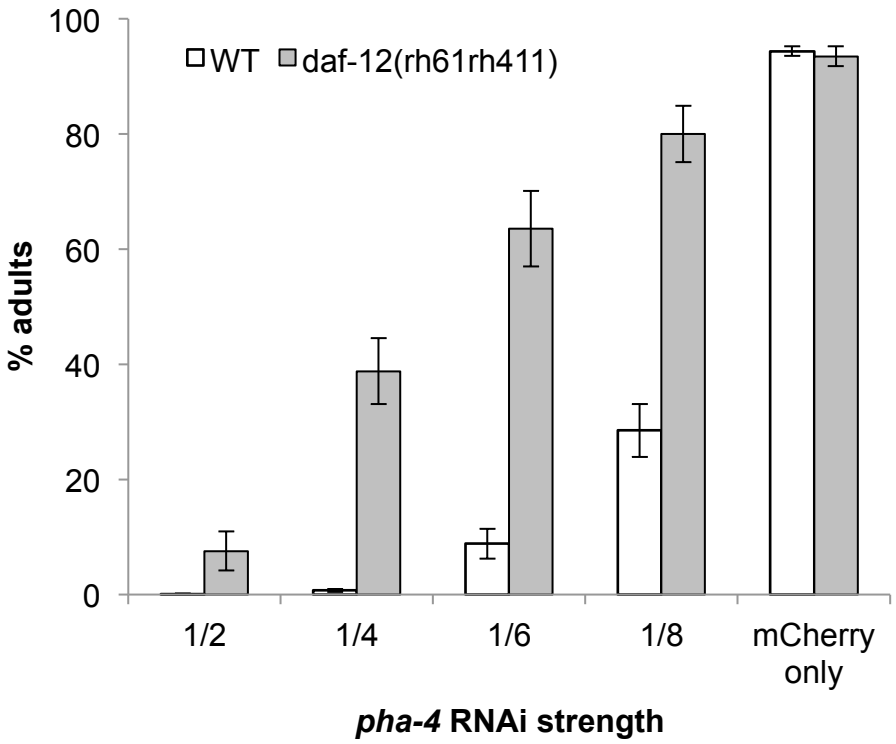


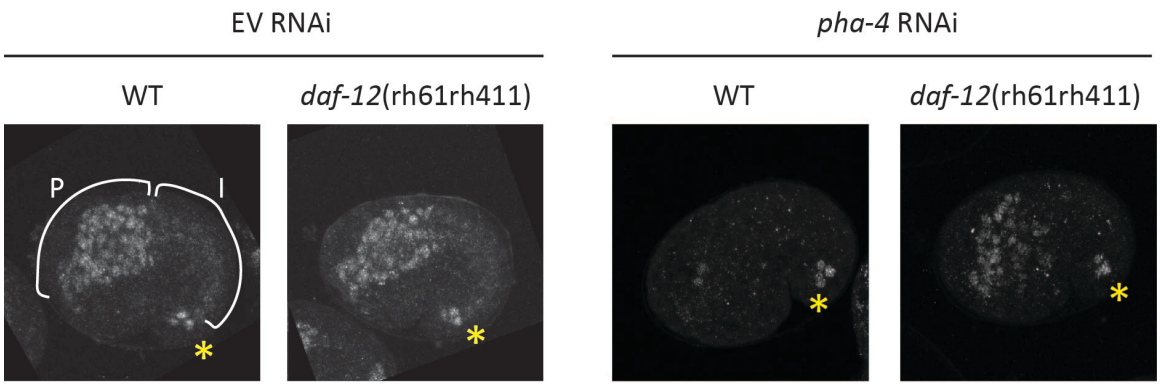
Figure 2.2. DAF-12 is required for robust *pha-4* RNAi.

WT and *daf-12*(rh61rh411; null) mutants (P0) were fed with *pha-4* dsRNA-expressing bacteria diluted with different amounts of mCherry (neutral) dsRNA-expressing bacteria. The fraction of progeny (F1) that grew to adulthood was scored. n=4. Error bars represent standard deviation.

Figure 2.3. PHA-4 accumulation was higher in *daf-12* mutants than in wildtype when treated with *pha-4* RNAi.

(A) Embryos of animals treated with EV (control; empty vector) or *pha-4* RNAi were collected and subjected to immunochemistry. Embryos at the 1.5 fold stage (mid-embryogenesis) were imaged for quantification. Normally, PHA-4 is expressed strongly in pharyngeal cells (P) and weakly in the intestine (I) at this stage of embryogenesis. Asterisks denote the rectal cells, which express *pha-4* regardless of *pha-4* RNAi. (B) PHA-4 in the anterior region of embryos was quantified as described in Materials and Methods. n=3. Student t-test was used for the analysis. a.u, arbitrary unit.

A



B

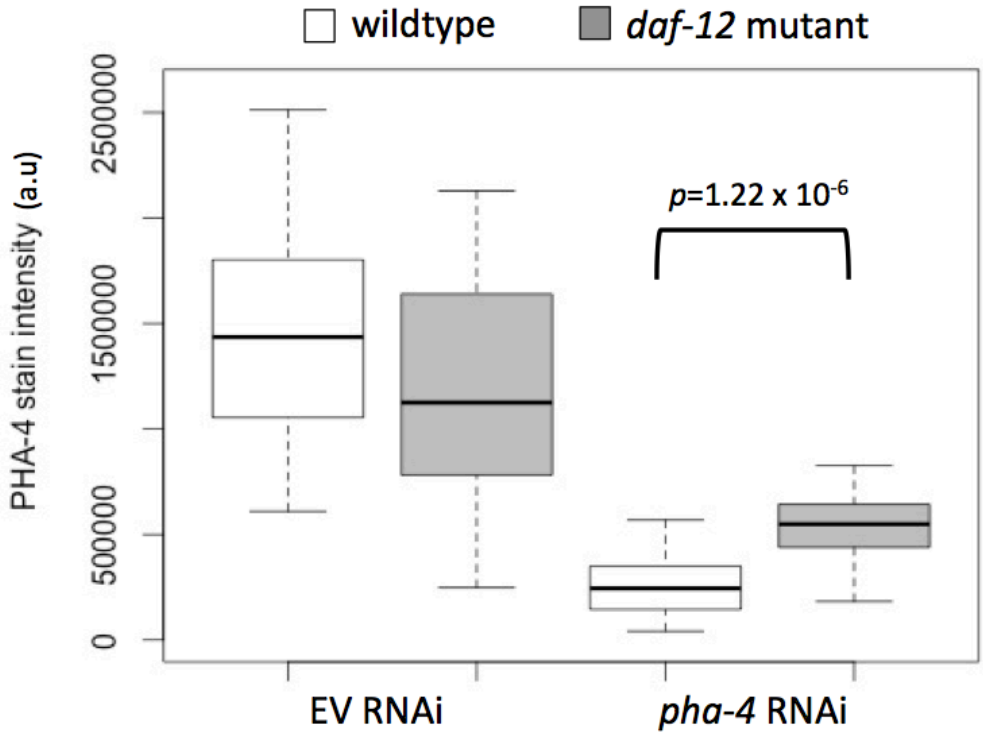


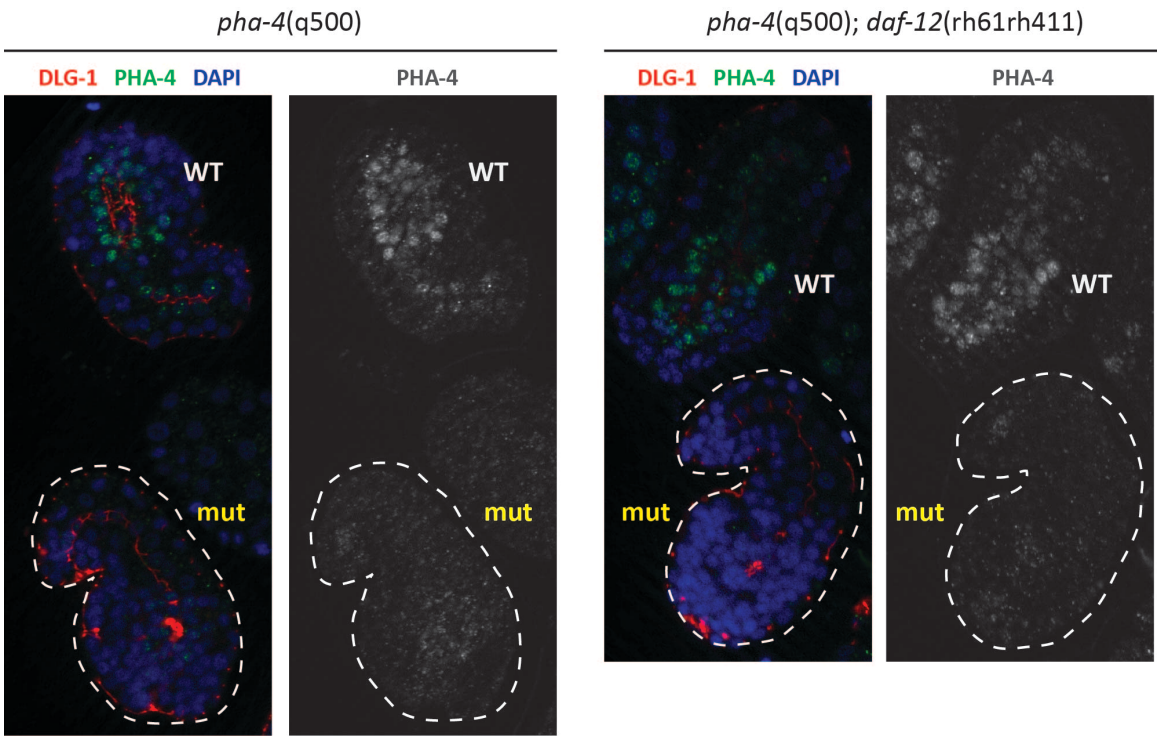
Figure 2.3 (continued). PHA-4 accumulation was higher in *daf-12* mutants than in wildtype when treated with *pha-4* RNAi.

How could DAF-12 increase the efficiency of *pha-4* RNAi? One possible explanation is that DAF-12 modulates the RNAi process and enhances the silencing efficiency. Another possibility is that DAF-12 acts as a suppressor of *pha-4* itself. In the latter case, the repression on *pha-4* would be lifted in *daf-12* mutants, and as a consequence, *pha-4* RNAi would appear less efficient due to a higher baseline of *pha-4* expression or activity in *daf-12* mutants. To differentiate between these two possibilities, we decided to determine whether DAF-12 regulates the lethality caused by *pha-4* partial loss-of-function mutations. If DAF-12 were a novel *pha-4* suppressor, lack of functional DAF-12 should increase the expression or the activity of PHA-4, thus restoring pharyngeal development in *pha-4* mutants. Alternatively, *daf-12* might only repress loss of function of *pha-4* achieved by RNAi. We found the later scenario to be true: a *daf-12* null mutation failed to restore PHA-4 protein in embryos harboring a *pha-4*(q500) partial loss-of-function mutation (Fig. 2.4A). Moreover, a *daf-12* mutation did not rescue the pharyngeal defects of *pha-4*(ts) mutants (KALTENBACH *et al.* 2005; UPDIKE and MANGO 2007) that were reared at semi-permissive temperature (Fig. 2.4B). The same result was obtained using a weaker *pha-4* partial loss-of-function allele, *zu225* (Fig. 2.5) (KALTENBACH *et al.* 2005). These results indicate that a weak *pha-4* RNAi phenotype in *daf-12* mutants is likely due to reduced activity of the RNAi process in the *daf-12* mutants, and not to modulation of *pha-4* itself.

Figure 2.4. *pha-4*(q500)-associated larval lethality and low PHA-4 accumulation were not suppressed by a *daf-12* null mutation.

(A) *pha-4*(q500) and *pha-4*(q500)*; daf-12*(rh61rh411) embryos were stained with PHA-4 and DLG-1 antibodies. DLG-1/Discs Large proteins are localized to the apical-basolateral boundary of epithelial cells, including pharyngeal and intestinal cells (BOSSINGER *et al.* 2001; FIRESTEIN and RONGO 2001). mut, the embryos homozygous for *pha-4*(q500); wt, the embryos whose genotypes were either *pha-4*(q500)/+ and +/+. (B) Previous studies reported that *smg-1*(cc546ts); *pha-4*(q500) incubated at 20°C from embryogenesis are arrested at the first larval stage (L1) (KALTENBACH *et al.* 2005; UPDIKE and MANGO 2007). The embryonic and larval lethality of progeny of *smg-1*(cc546ts); *pha-4*(q500) and *smg-1*(cc546ts); *pha-4*(q500)*; daf-12*(rh61rh411) incubated at 20°C was compared. n=2.

A



B

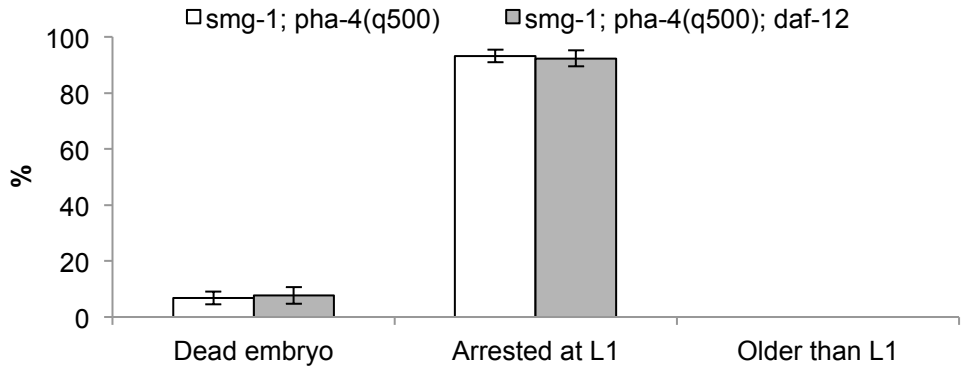
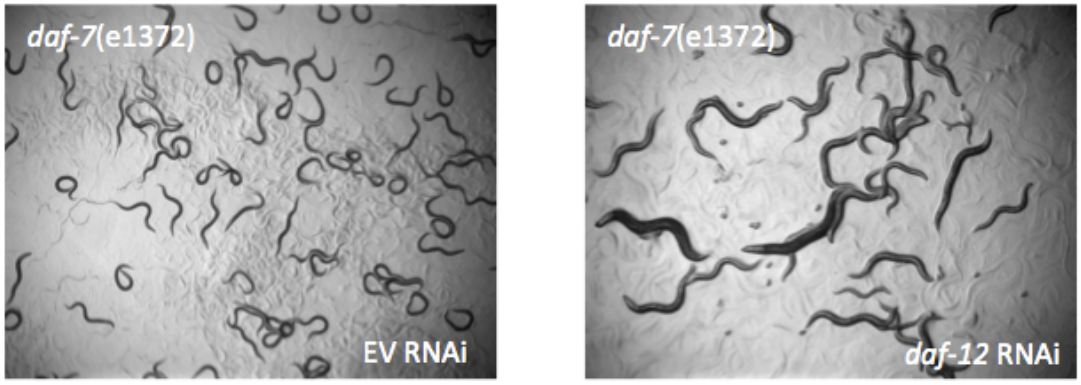


Figure 2.4 (continued). *pha-4*(q500)-associated larval lethality and low PHA-4 accumulation were not suppressed by a *daf-12* null mutation.

Figure 2.5. *daf-12* RNAi and a *daf-12* null mutation failed to rescue *pha-4*(zu225)-associated lethality.

(A) When cultured at 25°C, *daf-7*(e1372) animals became dauers, whereas *daf-12* RNAi inhibited the dauer-constitutive phenotype of *daf-7*(e1372) animals. This result showed that *daf-12* RNAi we used for the study was functional. EV RNAi, empty vector (control) RNAi. (B) *smg-1(cc546ts)*; *pha-4(zu225)* were fed with GFP RNAi (negative control), *smg-8* (positive control) (ROSAINS 2012), and *daf-12* RNAi food and cultured at 20°C, where the strains normally fail to form pharynx due to insufficient accumulation of PHA-4 proteins and die after hatching (KALTENBACH *et al.* 2005; UPDIKE and MANGO 2007). Error bars represent standard deviation. n=2. (C) A triple mutant *smg-1(cc546ts)*; *eri-8*(tm1860) *pha-4(zu225)* was constructed to increase the RNAi efficiency. The pharyngeal phenotypes of the triple mutants treated with either EV RNAi or *daf-12* RNAi were observed. Defects in pharynx further scored include pharynx unattached to the mouth, pharynx unattached to the intestine and no pharynx structure. (D) Pharynx phenotypes (normal pharynx, Pun (pharynx unattached to the mouth), and no pharynx) of triple mutant larvae, *smg-1(cc546ts)*; *pha-4(zu225); daf-12*(rh61rh411), reared at 19°C, 20°C and 21°C were observed. More than 80 larvae were examined except for the experiments at 19°C (28 larvae observed). n=1 for each temperature. (E) Although *smg-1(cc546ts)*; *pha-4(zu225); daf-12*(rh61rh411) that were reared at 21°C did not show any severe defects in pharynx, they failed to pass the first larval stage, whereas *smg-1(cc546ts)*; *pha-4(zu225)* grew to adulthood. Asterisks mark the mothers that were maintained at the permissive temperature (24°C) and produced progeny at 21°C.

A



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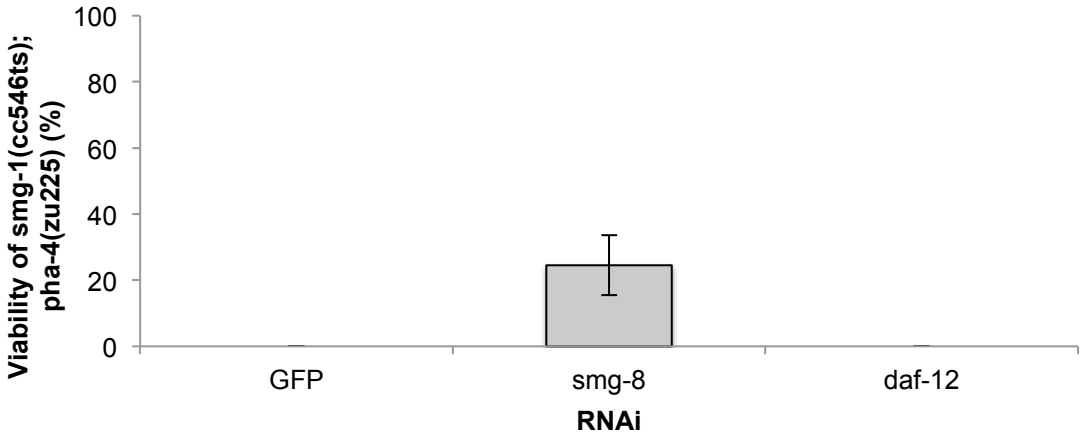

Figure 2.5 (continued). *daf-12* RNAi and a *daf-12* null mutation failed to rescue *pha-4*(zu225)-associated lethality.

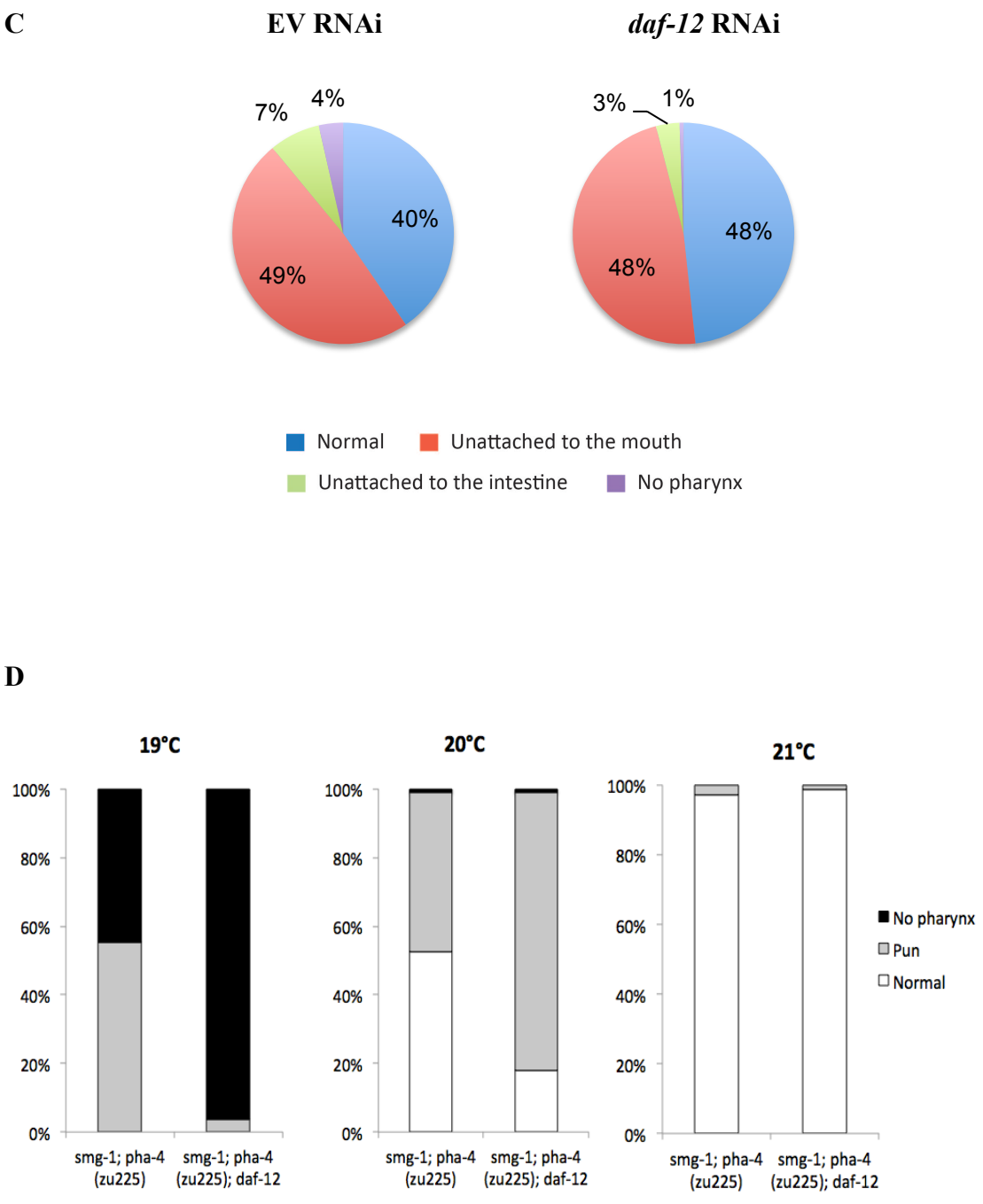

Figure 2.5 (continued). *daf-12* RNAi and a *daf-12* null mutation failed to rescue *pha-4*(zu225)-associated lethality.

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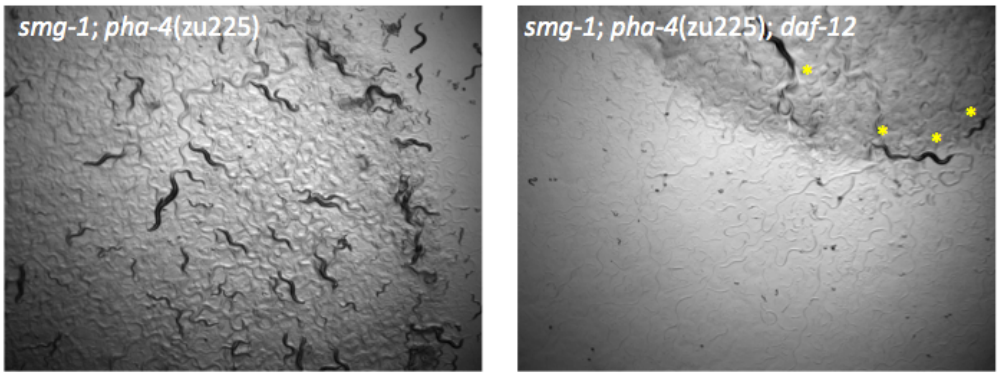

Figure 2.5 (continued). ***daf-12*** **RNAi and a** ***daf-12*** **null mutation failed to rescue** ***pha-4*****(zu225)-associated lethality.**

The role of DAF-12 in pha-4 RNAi regulation is distinct from that in the dauer pathway and heterochronic pathway.

DAF-12 is known for its role in developmental timing (ANTEBI *et al.* 1998; BETHKE *et al.* 2009; HAMMELL *et al.* 2009). Once hatched, *C. elegans* undergo four larval stages (L1 to L4) and become adults. Defects in the heterochronic pathway, which controls temporal development, lead to abnormal phenotypes in stage-specific processes. DAF-12 is essential for entry into dauer, an alternative larval stage where worms cease to undergo normal development and become resistant to environmental insults (CASSADA and RUSSELL 1975; FIELENBACH and ANTEBI 2008).

Depending on the positions of the molecular lesions, different *daf-12* mutants show distinct dauer phenotypes and heterochronic phenotypes (Table 2.1). Antebi and his colleagues have extensively characterized various *daf-12* mutants and grouped them into six different classes based on their dauer and heterochronic phenotypes (Table 2.1) (ANTEBI *et al.* 1998, 2000). For instance, the *daf-12* null mutants that were tested for *pha-4* RNAi repression (Fig. 2.2) were defective in dauer formation even in a harsh environment, but exhibited no particular heterochronic phenotype (ANTEBI *et al.* 1998). *daf-12*(rh61) mutants typically do not become dauer larvae, but show strong heterochronic phenotypes (ANTEBI *et al.* 1998). *daf-12* alleles in class 6 are unique in that the mutants become dauers constitutively (ANTEBI *et al.* 2000). We took advantage of these comprehensive descriptions of available *daf-12* mutants and asked whether a reduction in the *pha-4* RNAi efficiency in *daf-12* mutants tracked with either the dauer or heterochronic phenotype. Except for *daf-12*(rh173) mutants, which did not produce enough embryos for analysis, *daf-12* mutants from each class showed higher viability

Table 2.1. *daf-12* mutants with different dauer and heterochronic phenotypes.

Modified from Antebi *et al*. (2000).

Class	The allele used for the *pha-4* RNAi	Mutation	Location	Dauer phenotype	Heterochronic phenotype	
					Extra seam cells[a]	Gonadal migration defects[b]
1	rh257	Y699 splice acceptor	LBD	Defective	67%-100%	67%-100%
2	rh62rh157	S70stop, R564C	Before DBD, LBD	Defective	67%-100%	0%
3	rh61rh411	L147stop, Q618stop	DBD, LBD	Defective	0%	0%
4	rh193	G582K	LBD	Normal	34%-66%	34%-66%
5	rh284	P746S	LBD	Normal	0%	67%-100%
6	rh274	R564C	LBD	Constitutive	0%	67%-100%

[a] Seam cells (or lateral hypodermal cells) undergo cell divisions at the L2, L3, and L4 stages and become terminally differentiated (SULSTON *et al.* 1983; KIPREOS 2005). Defects in larval developmental timing results in production of fewer or extra seam cells.

[b] Normally, the gonad arms that have extended along the ventral muscle turn dorsally at the mid-L3 stage (HEDGECOCK *et al.* 1987).

than wildtype animals when treated with *pha-4* RNAi (Fig. 2.6). This result suggests that the effect of DAF-12 on robust silencing induced by RNAi is separable from the function of DAF-12 in dauer formation and developmental timing.

To uncouple the role of DAF-12 in *pha-4* RNAi regulation from dauer formation, we also tested whether the insulin-like pathway regulates *pha-4* RNAi efficiency. The insulin-like pathway is one of the environmental sensing pathways in *C. elegans*, and low activity of insulin signaling leads to constitutive dauer formation (GOTTLIEB and RUVKUN 1994; MALONE and THOMAS 1994; KIMURA *et al.* 1997; HU 2007). Two lines of evidence suggest that the insulin pathway does not affect *pha-4* RNAi efficiency. A previous work in the Mango lab showed that worms carrying a mutation in *daf-2* (Insulin/IGF receptor), which are arrested as dauers constitutively, are as sensitive to *pha-4* RNAi as wildtype animals (SHEAFFER *et al.* 2008). We also found that DAF-16/FOXO, a crucial downstream forkhead transcription factor in insulin-like pathway, did not alter *pha-4* RNAi sensitivity (Fig. 2.7). Together, these findings indicate that the *pha-4* RNAi efficiency is distinct from dauer-inducing pathways.

The effect of DAF-12 on pha-4 RNAi enhancement depends on ligands

One of the common characteristics of nuclear hormone receptors is that they activate transcription in a ligand-dependent manner. The activity of DAF-12 is also dependent on its ligands, dafachronic acids (DA) (MOTOLA *et al.* 2006; MAHANTI *et al.* 2014). Dietary cholesterol is serially oxidized to DA, and this process requires a complex network of enzymes (MAHANTI *et al.* 2014). NHR-8, another nuclear hormone receptor in *C. elegans*, activates the expression of *daf-36*/Rieske oxygenase, which catalyzes the first

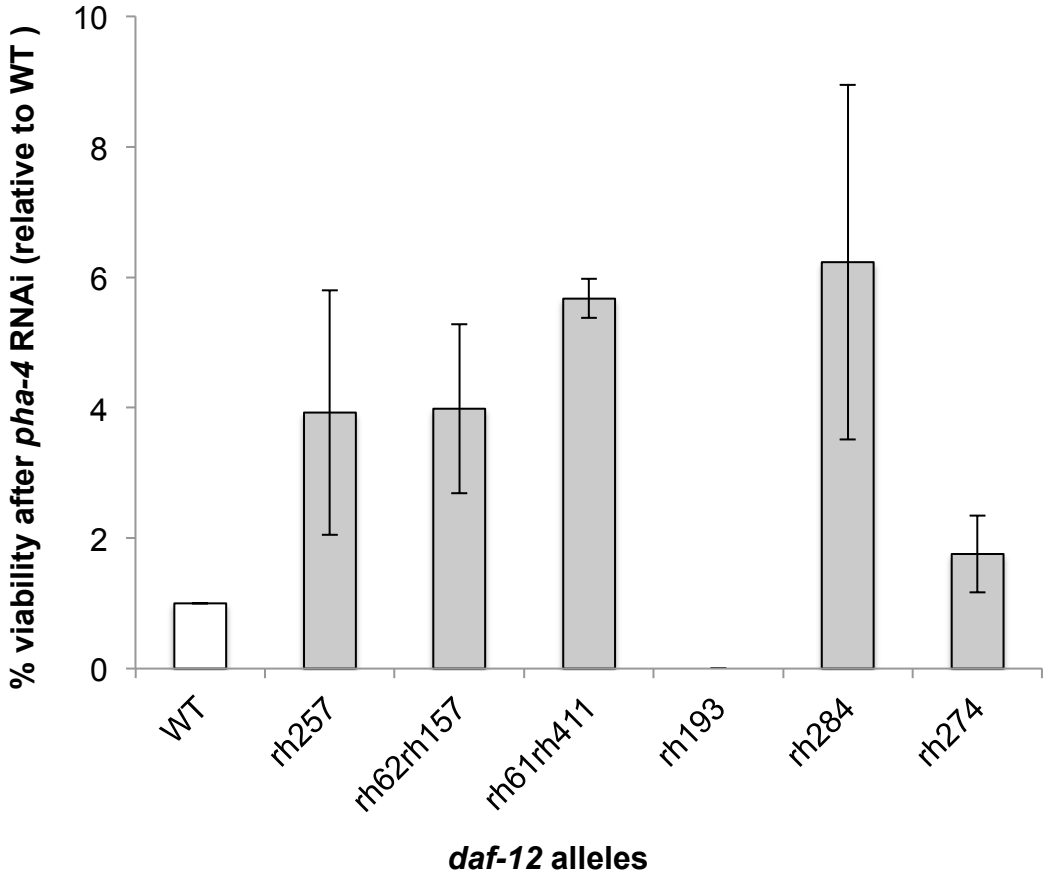

Figure 2.6. Animals bearing different types of mutations in *daf-12* all exhibited weak *pha-4* RNAi phenotypes.

The viabilities of different *daf-12* mutants (Table 2.1) were normalized to that of wildtype. n=2. Error bars represent standard deviation.

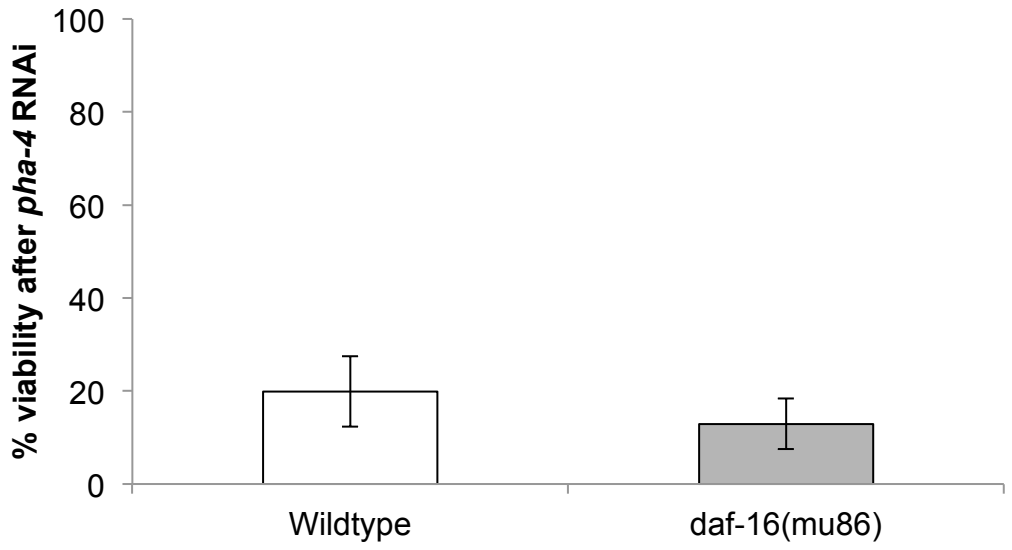

Figure 2.7. DAF-16 does not regulate the efficiency of *pha-4* RNAi.

Wildtype animals and *daf-16*(mu86) worms were fed with *pha-4* dsRNA-expressing bacteria from the L4 stage and the viability of their embryos were examined. n=3. Error bars represent standard deviation.

step of DA biosynthesis (MAGNER *et al.* 2013). Thus, *nhr-8* mutants exhibit the phenotypes associated with a low level (but not a complete loss) of DA when cultivated at an elevated temperature or without a cholesterol supplement (MAGNER *et al.* 2013). When treated with *pha-4* RNAi, only 6.8% of *nhr-8* mutants were viable, whereas 33.5% of wildtype worms grew to adulthood, indicating that *pha-4* RNAi was stronger when NHR-8 was inactivated and DA levels were low (Fig. 2.8). However, the viability of the double mutant, *nhr-8*(ok186); *daf-12*(rh61rh411), was reversed to 48.2% ± 10.8, which was virtually as high as that of *daf-12* single mutants (65.5% ± 14.0) (Fig. 2.8). This epistasis analysis suggests that a high level of unliganded DAF-12 can enhance *pha-4* RNAi.

The exacerbated *pha-4* RNAi phenotype in *nhr-8* mutants raised the question of whether DIN-1, which is the co-repressor of DAF-12 and competes with DA to bind to DAF-12 (MOTOLA *et al.* 2006), would be involved in *pha-4* RNAi-mediated silencing. Like *daf-12* mutants, *din-1* mutants were more viable than wildtype when treated with *pha-4* RNAi (Fig. 2.9), which implies that DIN-1 is also required for robust *pha-4* RNAi. Overall, these findings demonstrate that in wildtype animals, a high concentration of unliganded DAF-12 or DAF-12 bound to DIN-1 strengthens silencing triggered by RNAi.

We showed above that, regardless of the nature of mutation, various *daf-12* mutants were highly viable after *pha-4* RNAi. Except DAF-12(rh61), which is known to bind to DIN-1 weakly than wildtype DAF-12 (LUDEWIG *et al.* 2004), the extent to which mutations alter the affinity to DA and/or DIN is not know yet. Based on our observation that DIN-1 is required for robust RNAi, it is plausible that *daf-12* mutations lead to a

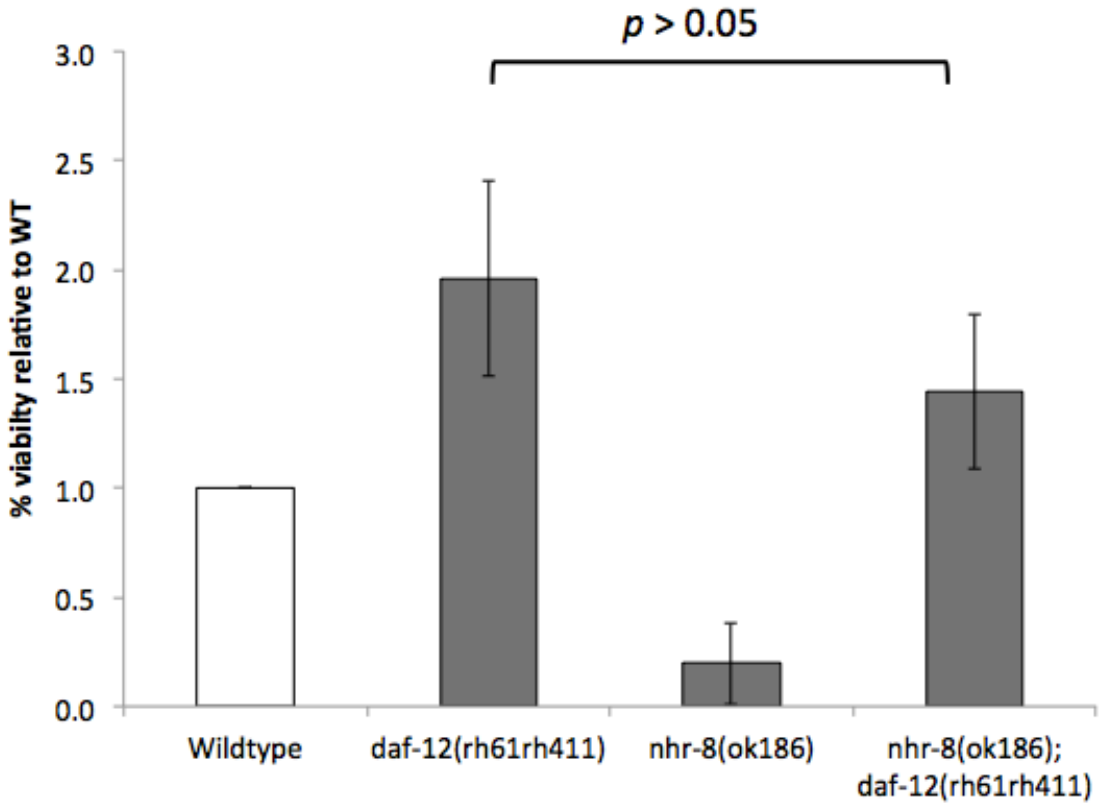

Figure 2.8. *daf-12* is epistatic to *nhr-8* in the *pha-4* RNAi regulation.

Animals at the L4 stage were treated with *pha-4* RNAi and the numbers of their progeny that passed the L1 stage were scored. The viability of each mutant was normalized to that of wildtype. Student t-test shows that the viability of *daf-12* mutants was not significantly different from that of *nhr-8*; *daf-12*. n=4. Error bars represent standard deviation.

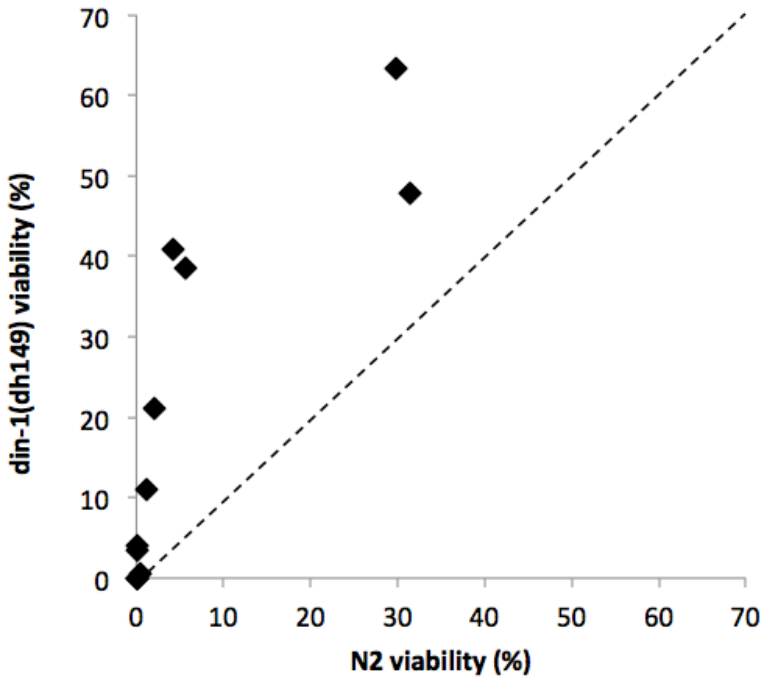

Figure 2.9. *pha-4* (RNAi)-associated lethality was repressed in *din-1*(dh149) mutants. N2 (wildtype) and *din-1*(dh149) animals were treated with *pha-4* RNAi, and the fractions of their progeny that grew to adulthood were scored. The dotted line with the slope of one is superimposed to the graph to help the comparison between the viabilities of N2 and *din-1* mutants. n=4.

slight decrease in the affinity to DIN-1, which results in low RNAi efficiency in *daf-12* mutants.

DAF-12 promotes a decrease in Pol II occupancy at the locus targeted by RNAi

How could DAF-12 regulate the robustness of RNAi? DAF-12 could increase the efficiency of post-transcriptional silencing induced by RNAi. Alternatively, DAF-12 might strengthen RNAi-triggered transcriptional inactivation, a process recently discovered and that depends on NRDE (Nuclear RNAi-defective) factors (GUANG *et al.* 2008). In *C. elegans*, siRNAs are incorporated into the nuclear argonaute NRDE-3, which localizes to the nucleus and recruits other nuclear proteins to stall RNA polymerase II during transcriptional elongation of the targeted gene (GUANG *et al.* 2010; BURKHART *et al.* 2011). Of note, the NRDE pathway is essential for RNAi targeting only a subset of genes (GUANG *et al.* 2008), indicating that transcriptional silencing is not universally required for RNAi-induced silencing.

To determine whether DAF-12 prevents a change in the level of Pol II bound to the target gene upon RNAi, we obtained a pool of mixed-stage embryos (Fig. 2.10A) from *pha-4* RNAi-treated worms and performed Pol II chromatin immunoprecipitation (ChIP) followed by qPCR. We found that wildtype and *daf-12* null mutants have different patterns of Pol II occupancy at *pha-4* in response to *pha-4* RNAi (Fig. 2.10D and E). In wildtype embryos, Pol II enrichment at the *pha-4* locus decreased by half throughout the gene body after *pha-4* RNAi (Fig. 2.10D and E), indicating that transcriptional initiation, not just elongation, was inhibited. In contrast to wildtype, *daf-12* mutants failed to show a

Figure 2.10. DAF-12 is required for transcriptional repression of *pha-4* induced by RNAi.

(A) Distribution of stages of embryos used for chromatin immunoprecipitation (ChIP) Wildtype (WT) and *daf-12* mutants were treated with EV (empty vector; control) RNAi or *pha-4* RNAi and then mixed stages of their embryos were subjected to ChIP. More than 140 embryos were observed for each strain and each RNAi treatment. (B) *pha-4* gene structure. Grey boxes represent eight exons of *pha-4*. The sites amplified by qPCR primers are indicated by red bars. The blue line denotes the region targeted by *pha-4* dsRNA. (C, D and E) qPCR analysis of Pol II ChIP at four different regions of *pha-4* (indicated by red bars in (A)) in wildtype and *daf-12* mutants. As a negative control, we also measured Pol II enrichment at *srw-99* (C), which encodes a serpentine receptor. We used two positive controls, *eft-3*, a housekeeping gene encoding a translation elongation factor, and *elp-1*, a gene located 5kb downstream of *pha-4* and encodes a microtubule-interacting protein (C). Pol II enrichment at the *pha-4* locus and control genes was calculated relative to input DNA (% input). Then, % input at *pha-4* was rescaled using Pol II enrichment at *srw-99* and *eft-3* (D) or *elp-1* (E) as 0 and 1, respectively. The ratio of Pol II occupancy after *pha-4* RNAi to EV RNAi treatment is shown. n=3. Error bars represent standard deviation.

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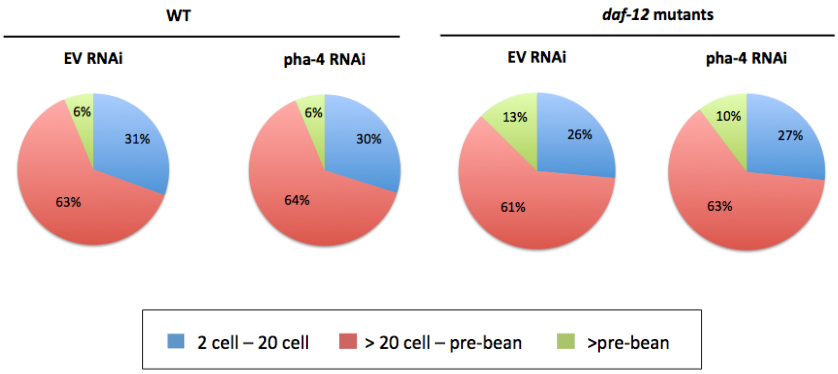

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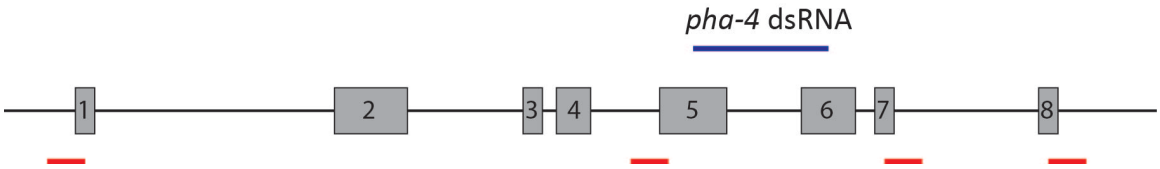

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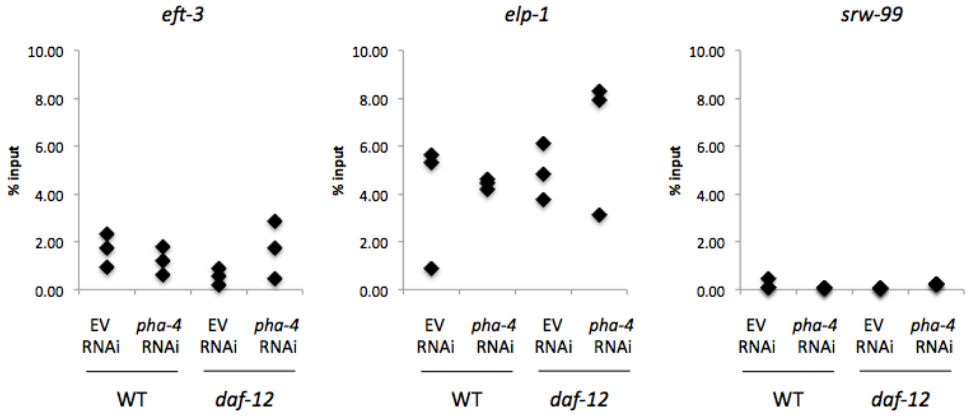
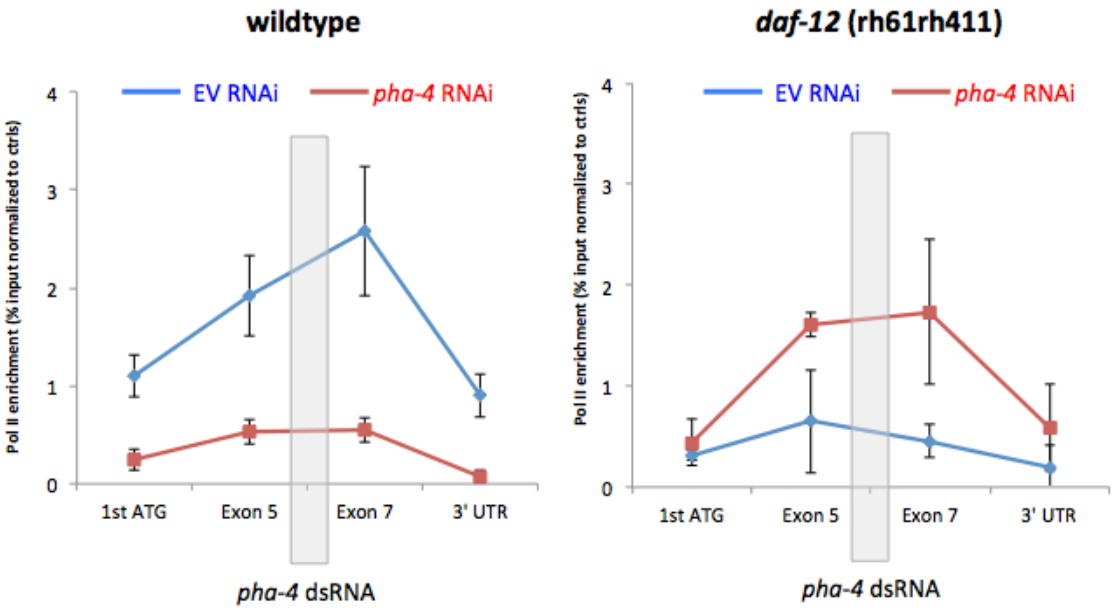


Figure 2.10 (continued). DAF-12 is required for transcriptional repression of *pha-4* induced by RNAi.

D. Normalized to *eft-3* and *srw-99*



E. Normalized to *elp-1* and *srw-99*

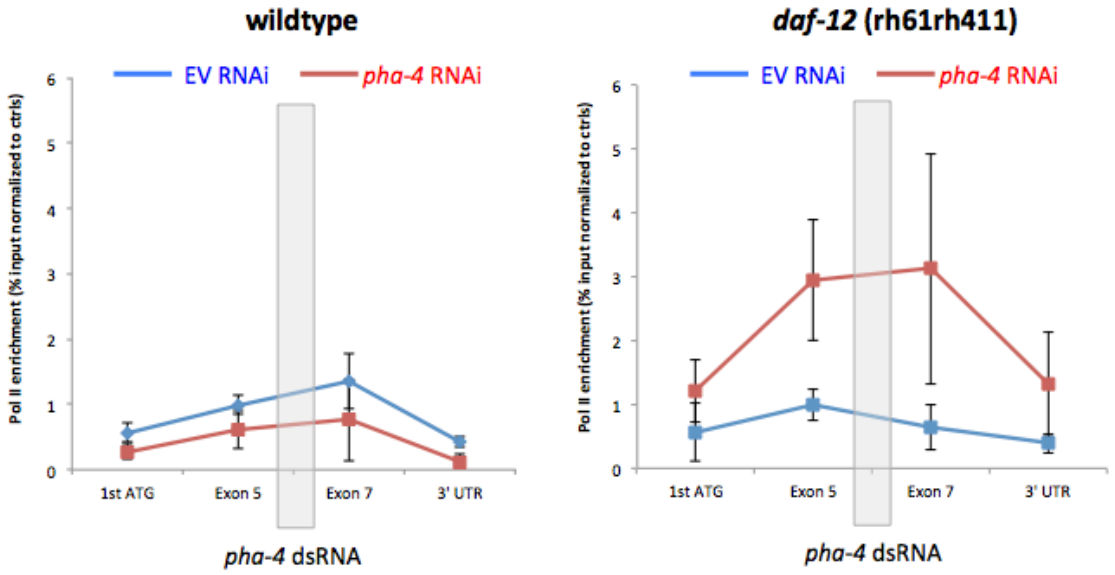


Figure 2.10 (continued). DAF-12 is required for transcriptional repression of *pha-4* induced by RNAi.

significant reduction in Pol II enrichment (Fig. 2.10D and E). Instead, Pol II occupancy at the *pha-4* locus in *daf-12* mutants increased when treated with *pha-4* RNAi (Fig. 2.10D and E). These results suggest that lack of RNAi-induced transcriptional repression in *daf-12* mutants is associated with their low RNAi efficiency. We used two different reference genes (*eft-3* and *elp-1*) to normalize Pol II and the analyses produced the same trends, although the absolute values varied (Fig. 2.10D and E). Together, our Pol II-qPCR experiments revealed the association between increased Pol II occupancy and low silencing activity in *daf-12* mutants.

In addition to inhibition of transcription, RNAi promotes the enrichment of H3K9me3 at targeted loci (GUANG *et al.* 2010; BURTON *et al.* 2011; BUCKLEY *et al.* 2012; GU *et al.* 2012), a histone modification related to inactive transcription (VAKOC *et al.* 2005; BARSKI *et al.* 2007; MIKKELSEN *et al.* 2007; KIM *et al.* 2007; REGHA *et al.* 2007; ROSENFELD *et al.* 2009; BLACK and WHETSTINE 2011). Using H3K9me3 ChIP-qPCR, we asked whether the levels of H3K9me3 at the *pha-4* locus differ in wildtype versus *daf-12* mutants after *pha-4* RNAi. Unlike the distinct patterns of Pol II enrichment at the *pha-4* locus in wildtype and *daf-12* mutants, both strains showed a two-fold increase in H3K9me3 at the *pha-4* coding region (Fig. 2.11). This result derived from a single experiment and more experiments are required to draw any solid conclusion. However, if this pattern is reproducible, it would indicate that DAF-12 does not affect the accumulation of H3K9me3 in response to RNAi. In addition, it would suggest that DAF-12 could adjust Pol II loading independently of H3K9me3.

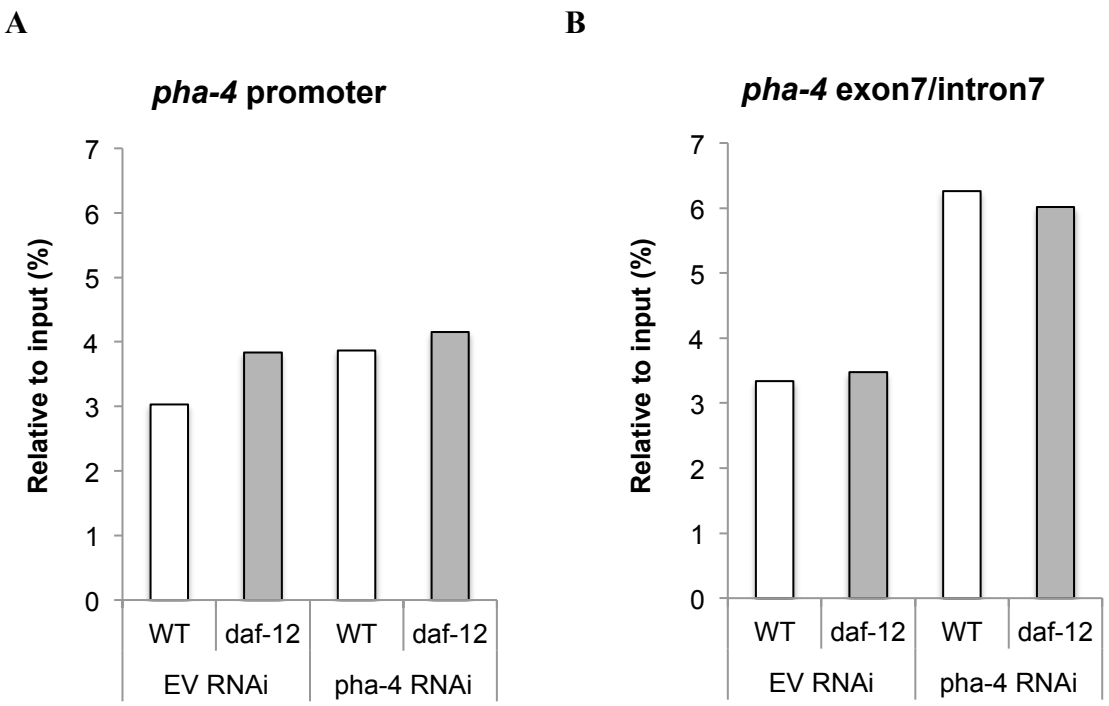


Figure 2.11. DAF-12 is not required for the H3K9me3 enrichment at the *pha-4* locus after *pha-4* RNAi.

H3K9me3, a histone modification associated with inactive transcription, is involved in maintenance of the silenced state induced by RNAi (BURTON *et al.* 2011; BUCKLEY *et al.* 2012; GU *et al.* 2012). Mixed stages of embryos of animals treated with empty vector (EV; control) RNAi and *pha-4* RNAi treatment were subjected to H3K9me3 chromatin immunopreciptation (ChIP). qPCR primers were designed to target *pha-4* promoter region (200bp upstream of the first ATG) (A) and *pha-4* exon 7 and intron 7 junction (B). % input values at the *pha-4* locus were normalized to H3K9me3 enrichment at *pcaf-1*. n=1.

DAF-12 acts in mothers to enhance the RNAi efficiency in zygotes

The NRDE pathway is more critical for RNAi-mediated silencing in progeny than P0 parents. As explained above, in our typical experiment, we initiated RNAi in hermaphrodites (P0) at the fourth (last) larval stage and examined RNAi phenotypes in embryos (F1). The effect of *daf-12* on RNA pol II accumulation raised the question of whether DAF-12 boosted the RNAi efficiency in the P0 animals that were directly exposed to dsRNA or functioned mostly in embryos to trigger transcriptional regulation, similar to NRDE-3.

To determine whether DAF-12 acted in dsRNA-exposed animals (P0) or their zygotes (F1), we fed *daf-12*/+ heterozygous animals (P0) with *pha-4* RNAi and tested whether the *pha-4* RNAi lethality of the F1 depended on the genotype of mothers or zygotes (F1). *daf-12* homozygous embryos (F1) from heterozygous animals (P0) displayed as strong a *pha-4* RNAi response as their wildtype siblings (Fig. 2.12). This result suggests that DAF-12 functions in P0 mothers, and that a wildtype copy of *daf-12* in the P0 was able to induce robust *pha-4* RNAi in zygotes. In this experiment, we used hermaphrodites, which can produce both sperm and oocytes. To test whether DAF-12 acted in mothers or fathers, we established crosses between wildtype parents and *daf-12* mutants, which revealed that DAF-12 was required in mothers to regulate *pha-4* RNAi in zygotes (Fig. 2.13). Together, these results indicate that functional DAF-12 in mothers is necessary and sufficient to enhance RNAi efficiency in zygotes.

If DAF-12 acts in the animals (P0) that are directly exposed to dsRNA-expressing bacteria, we reasoned that DAF-12 may be required for robust *pha-4* RNAi in the P0 as well. Low expression or activity of *pha-4* during larval development leads to defects in

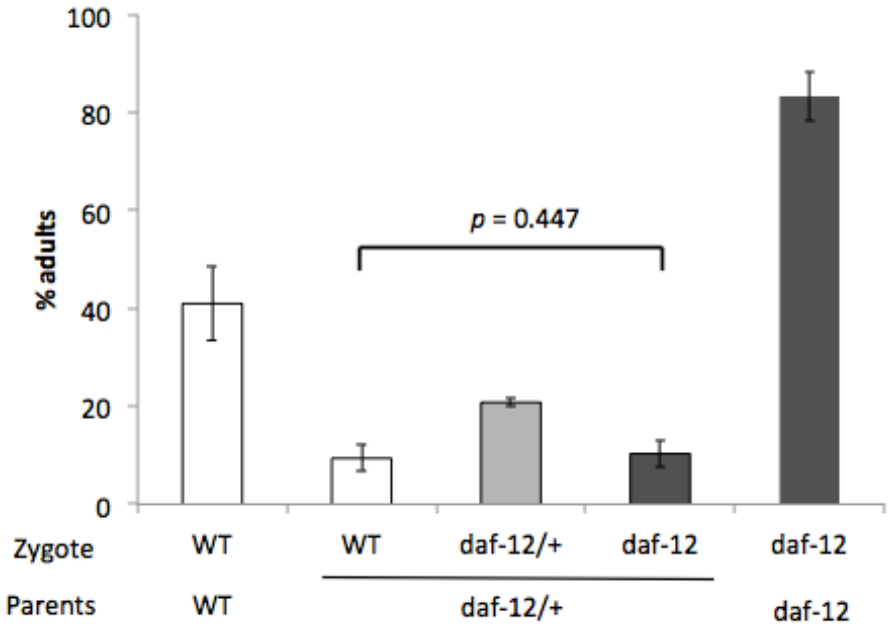


Figure 2.12. The genotype of parents, not zygotes, determines the severity of *pha-4* RNAi phenotypes in zygotes.

Animals with two copies of *daf-12* (WT), heterozygous for *daf-12* null mutation (*daf-12*/+) or homozygous for *daf-12* null mutation (*daf-12*) were treated with *pha-4* RNAi. The genotypes of the progeny of *daf-12* heterozygous animals were confirmed by PCR. Student t test indicates that wildtype and *daf-12* homozygous mutant siblings from heterozygous hermaphrodites were not significantly different in *pha-4*(RNAi)-associated lethality. n=3. Error bars represent standard deviation.

Figure 2.13. DAF-12 functions in mothers to enhance *pha-4*(RNAi) lethality in zygotes.

(A) Three pharynx phenotypes (normal, Pun and no pharynx) were scored to test the parental vs. maternal effect of DAF-12. Antibody staining for DLG-1/Disc large, which is localized to adherence junctions (BOSSINGER *et al.* 2001; FIRESTEIN and RONGO 2001), allowed us to better observe pharyngeal defects. mCherry expression indicated that the embryos were cross-progeny, not self-progeny. Asterisks denote the mouths of embryos. Yellow bar marks the pharyngeal-intestinal valve. (B) The *daf-12* null mutation from mothers was sufficient to rescue severe pharyngeal defects caused by *pha-4* RNAi in *daf-12* heterozygous progeny. The numbers in parentheses are the numbers of embryos examined for pharyngeal defects. Herma., hermaphrodites. n=3.

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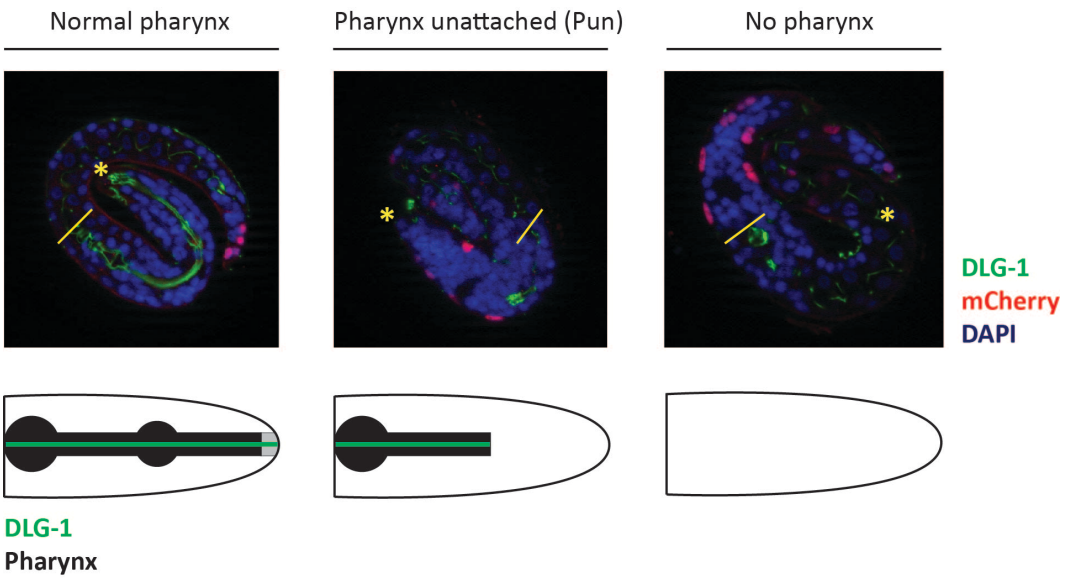

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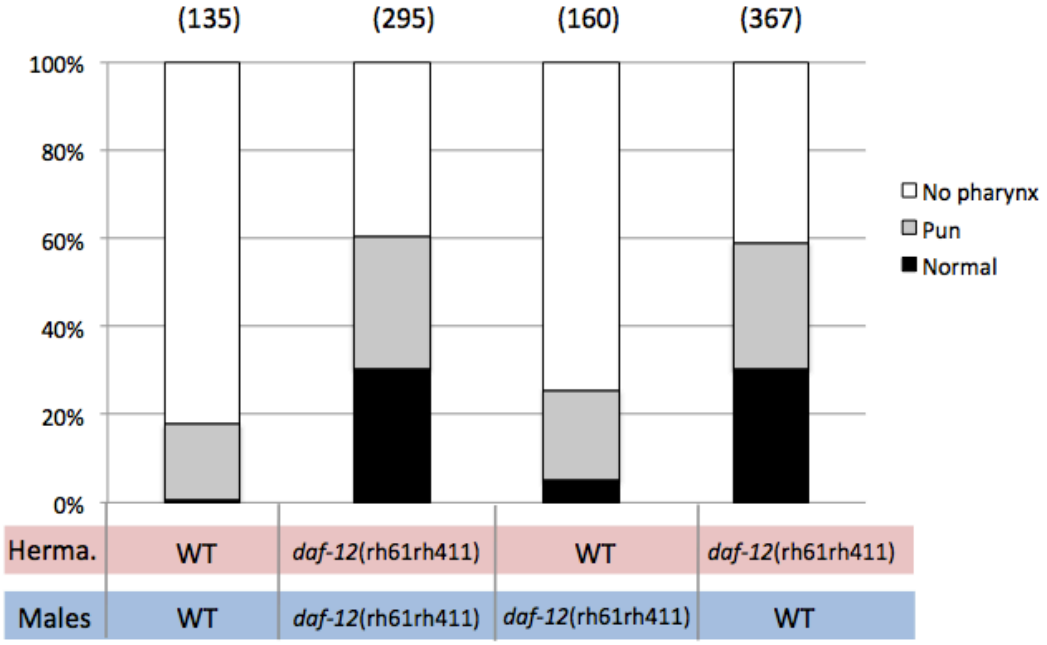

Figure 2.13 (continued). DAF-12 functions in mothers to enhance *pha-4*(RNAi) lethality in zygotes.

gonad development and sterility (Fig. 2.14A) (UPDIKE and MANGO 2007; CHEN and RIDDLE 2008). P0 *daf-12* mutants that were exposed to *pha-4* RNAi from the first larval stage were as susceptible to *pha-4* RNAi as wildtype larvae (Fig. 2.14B), indicating that DAF-12 was dispensable for robust RNAi in P0 animals that were directly exposed to dsRNA. In summary, DAF-12 does not affect the RNAi efficiency in P0 mothers, but it is required for strong silencing in offspring. These observations demonstrate that unlike many regulators of RNAi, DAF-12 is involved in the transmission of silencing signals from mothers to zygotes.

The effect of DAF-12 on the RNAi enhancement depends on the population density during mother's larval development

A remarkable feature of DAF-12 is that its activity is determined by the quality of the environment; in a favorable environment, DAF-12 promotes reproductive growth, whereas harsh conditions result in a decrease in the level of DA, and unliganded DAF-12 promotes entry into dauer (NOLAN *et al.* 2002; GERISCH and ANTEBI 2004; FIELENBACH and ANTEBI 2008; HAMMELL *et al.* 2009). Thus, we investigated the contribution of the parental environment to DAF-12-mediated RNAi enhancement in zygotes. Our choice of the environmental input was high population density, which is known to induce dauer formation (GOLDEN and RIDDLE 1982). *C. elegans* produces and continuously secretes pheromones, and its concentration is indicative of population density (GOLDEN and RIDDLE 1984; BUTCHER *et al.* 2009). Overcrowding or a high level of pheromone dampens the biosynthesis of DA, and animals are committed to the dauer stage instead of

Figure 2.14. *daf-12* mutants are as susceptible to *pha-4* RNAi as wildtype (WT) during larval growth.

(A) *pha-4* RNAi during larval development cause defects in gonad development and leads to sterility (UPDIKE and MANGO 2007; CHEN and RIDDLE 2008). (B) Wildtype and *daf-12*(rh61rh411) worms were fed with *pha-4* dsRNA-expressing bacteria throughout larval development and their germ lines were examined when they became adults. The numbers in parentheses are the numbers of gonad arms examined for germ line defects. n=3.

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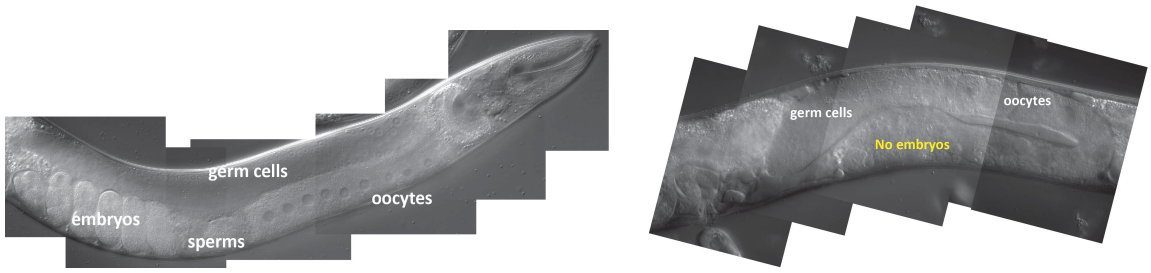

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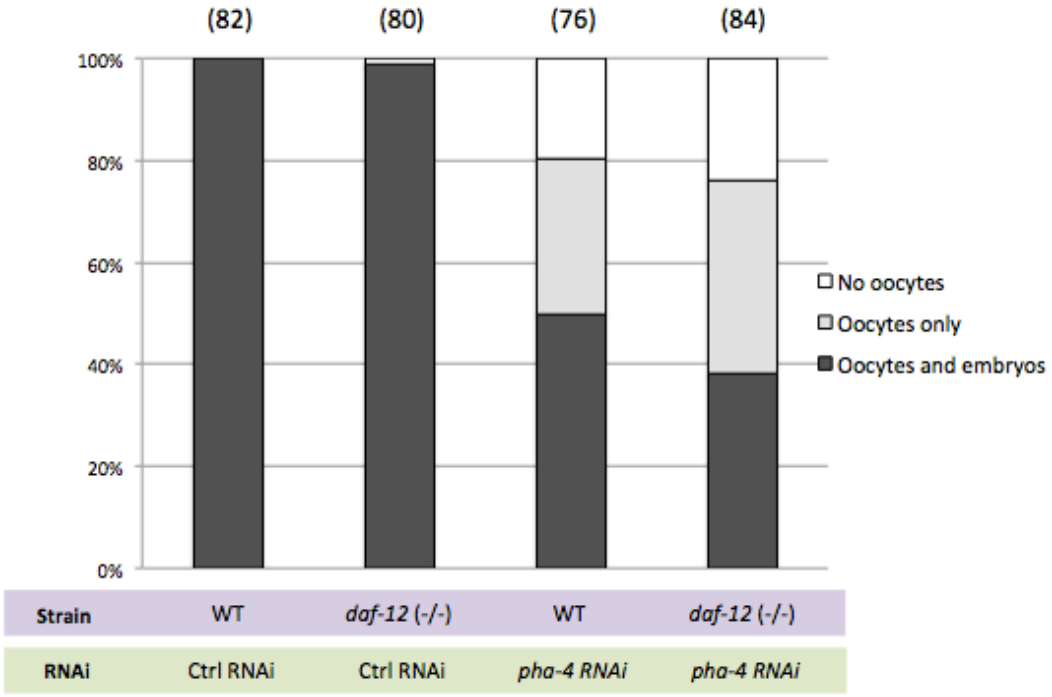


Figure 2.14 (continued). *daf-12* mutants are as susceptible to *pha-4* RNAi as wildtype (WT) during larval growth.

reproductive development (GOLDEN and RIDDLE 1982; SCHACKWITZ *et al.* 1996; SCHAEDEL *et al.* 2012). We cultivated wildtype and *daf-12* mutants under crowded (~1500 worms) versus sparse (~60 worms) conditions during their larval development and then treated the same number of worms from each condition with *pha-4* RNAi (Fig. 2.15A). Worms were never starved or became dauers during the procedure. Strikingly, we found that when mothers developed in a low-population condition and were treated with *pha-4* RNAi, the viability of *daf-12* mutants was 2.5 fold higher than that of wildtype (Fig. 2.15B), similar to what we observed previously. In contrast, the strong RNAi phenotype was restored in *daf-12* mutant embryos when their mothers were cultivated under the crowded condition (Fig. 2.15B). These results suggest that the function of DAF-12 in RNAi depends on the environmental context; normally, when mothers sense a relatively low concentration of pheromone, maternal DAF-12 is required to ensure robust silencing in zygotes induced by RNAi. If mothers are exposed to a high dose of pheromone, DAF-12 becomes dispensable, and RNAi in zygotes remains robust by an unknown pathway. Of note, we found that *daf-12* mutants show a low RNAi response regardless of growth temperatures (20°C versus 25°C) (Fig. 2.16), implying that not all environmental conditions affect the dependency on DAF-12 in RNAi.

What pathways could mediate the environmental effect on RNAi? Based on the previous findings that the TGF-β pathway is regulated by environmental cues (REN *et al.* 1996; SCHACKWITZ *et al.* 1996), we examined the efficiency of *pha-4* RNAi for TGF-β pathway mutants (Fig. 2.17A). A mutation in *daf-4*, which encodes a type II TGF- β receptor, enhanced *pha-4* RNAi, whereas a mutation in *daf-3*, whose protein products act

Figure 2.15. The role of DAF-12 in RNAi depends on the environmental context during the larval development of mothers.

(A) A schematic diagram of the experimental design for testing the environmental contribution to RNAi. Wildtype and *daf-12* mutant animals were hatched and grew on either an under-crowded plate (Few; ~60 animals) or an over-crowded plate (Many; ~1500 animals). Food was added to the populated plates during the experiment so that worms were never starved. Then, animals at the fourth larval stage (L4) were treated *pha-4* RNAi under the same environment (15 worms/*pha-4* RNAi). The number of their progeny that grew to adulthood was scored. (B) The viability of animals was normalized to that of "few" WT. WT, wildtype; *daf-12* mut, *daf-12*(rh61rh411). The dotted grey line indicates the wildtype level viability, which is one. Solid gray lines represent the average values. n=4.

A

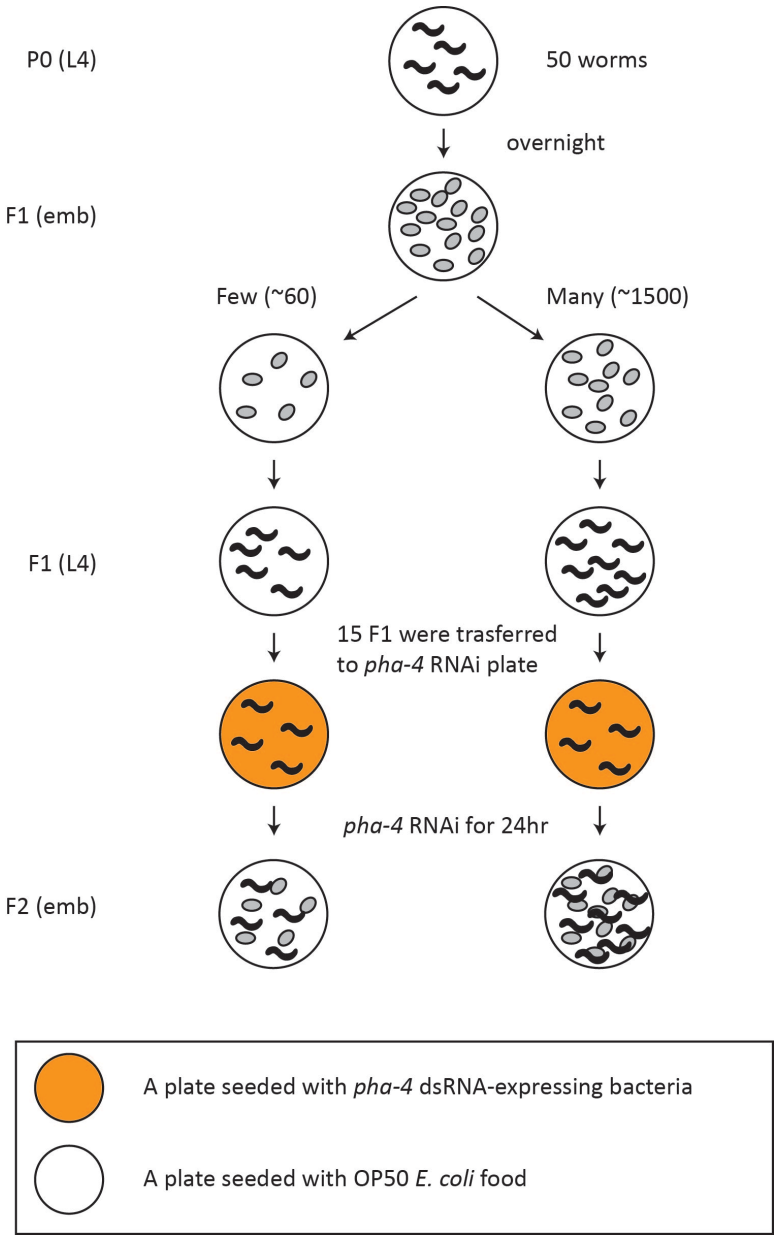


Figure 2.15 (continued). The role of DAF-12 in RNAi depends on the environmental context during the larval development of mothers.

B

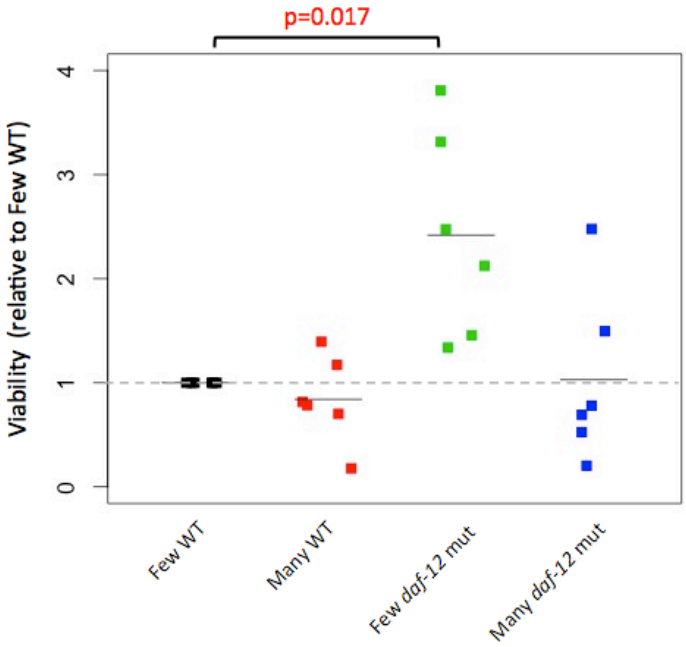


Figure 2.15 (continued). The role of DAF-12 in RNAi depends on the environmental context during the larval development of mothers.

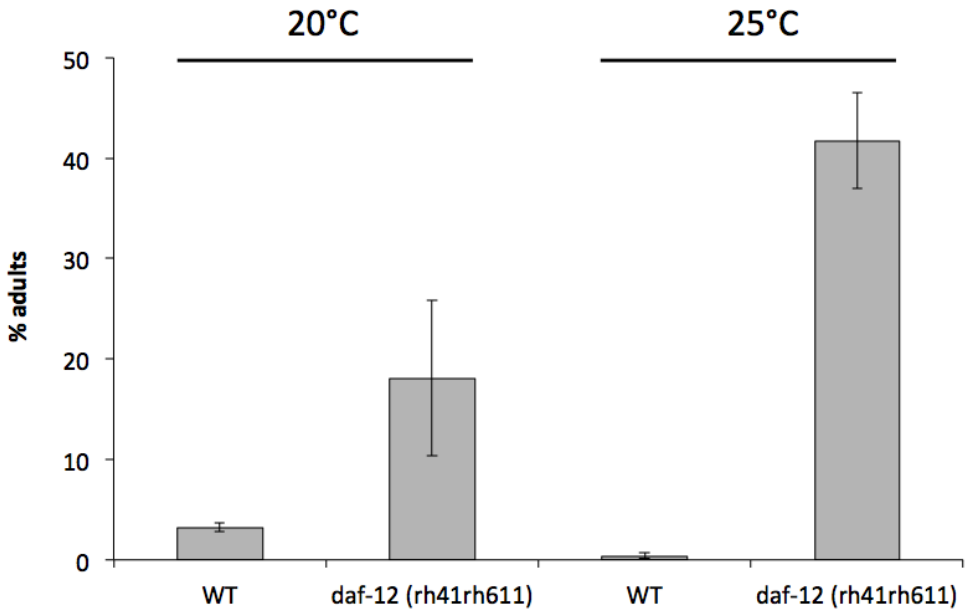


Figure 2.16. The effect of DAF-12 in RNAi enhancement does not depend on the growth temperature.

Wildtype (WT) and daf-12 mutants hatched and grew at either 20°C or 25°C. When the animals reached the L4 stage, they were treated with *pha-4* RNAi at 25°C. The numbers of progeny that grew to adulthood were scored. n=3. Error bars represent standard deviation.

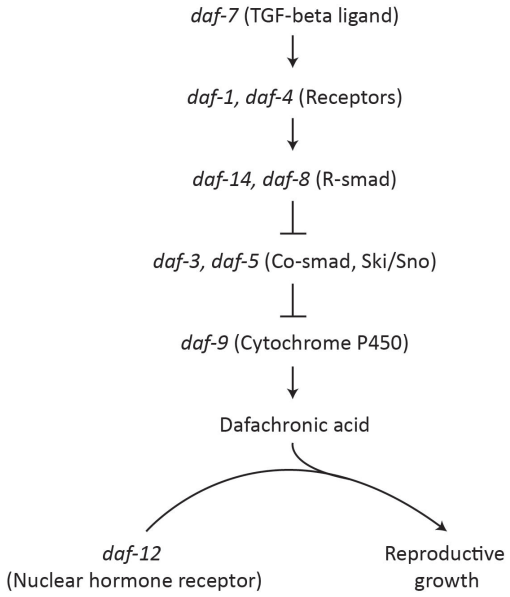
as a negative regulator of the TGF-β dauer pathway, repressed *pha-4* RNAi (Fig. 2.17B). We note here that all the mutants used for these assays were not backcrossed, therefore it is possible that the alteration in *pha-4* RNAi efficiency is due to some background mutations. Moreover, since the mutants were tested only with *pha-4* RNAi, it is not yet clear whether the TGF-β dauer pathway regulates RNAi in general or not. Nevertheless, the direction of enhancement and repression by *daf-4* and *daf-3* respectively is consistent with the notion that this pathway could mediate the environmental cues.

Among the mutants that showed changes in the efficiency of *pha-4* RNAi, *daf-4* mutants exhibited the strongest enhancement (Fig. 2.17B). *daf-4* mutants form dauer larvae constitutively (Daf-c) (GOLDEN and RIDDLE 1984) and the dauer phenotype is suppressed by *daf-12* mutations, indicating *daf-12* is downstream of *daf-4* in dauer pathway. However, our epistasis analysis revealed that *daf-4* was epistatic to *daf-12* in *pha-4* RNAi regulation (Fig. 2.18). Daf-c phenotype of *daf-4* mutants suggests that the animals activate mechanisms responding to unfavorable environments. Both populous conditions and the *daf-4* mutation induced strong *pha-4* RNAi response without DAF-12, which strengthens the argument that environmental conditions influence RNAi robustness. Whether DAF-4 mediates the environmental effect on RNAi remains to be seen, but this novel epistatic interaction between *daf-4* and *daf-12* may lead to identification of new mechanisms that elicit physiological responses to the environment.

Figure 2.17. Some mutants in the TGF-beta dauer pathway enhance or mitigate *pha-4* RNAi phenotype.

(A) TGF-beta dauer pathway in *C. elegans* (modified from (FIELENBACH and ANTEBI 2008)). (B) Various mutants in the TGF-beta dauer pathway were treated with *pha-4* RNAi and viabilities of their embryos were normalized to that of wildtype. n=3 for each mutant.

A



B

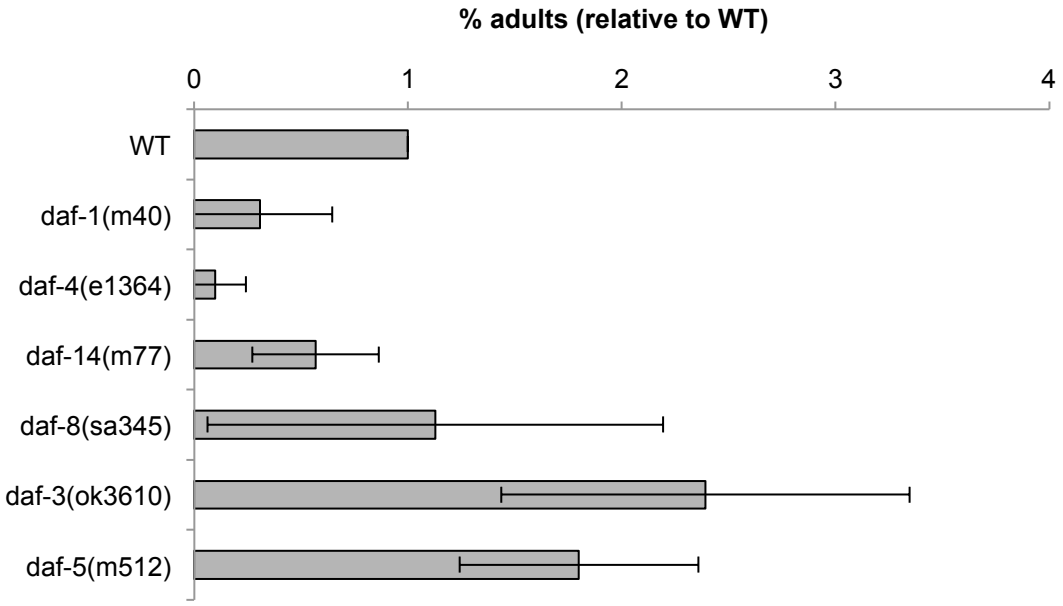


Figure 2.17 (continued). Some mutants in the TGF-beta dauer pathway enhance or mitigate *pha-4* RNAi phenotype.

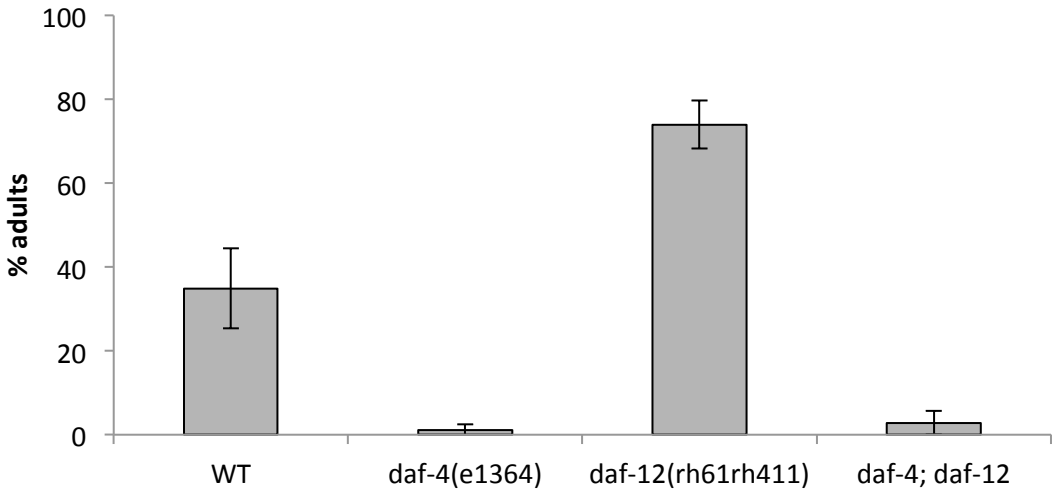


Figure 2.18. *daf-4* is epistatic to *daf-12* in *pha-4* RNAi regulation.

pha-4 RNAi causes lethality. Animals were treated with *pha-4* RNAi and the number of their progeny passed the first larval stage was scored. Error bars represent standard deviation. n=3. Error bars represent standard deviation.

The effect of DAF-12 on RNAi is not solely dependent on the NRDE pathway

How could DAF-12 in mothers ensure robust RNAi in zygotes? Independent microarray studies suggest that *daf-12* transcripts are not deposited in early embryos (BAUGH *et al.* 2003; REINKE *et al.* 2003; YUZYUK *et al.* 2009; LEVIN *et al.* 2012). Therefore, it is less likely that maternal DAF-12 protein is deposited in zygotes and establishes RNAi-triggered silencing. An alternative mechanism could be that DAF-12 in mothers regulates the maternal contribution of RNAi machinery components to zygotes. We decided to test a potential interaction between DAF-12 and the NRDE pathway, which is required to maintain RNAi silencing in the progeny of dsRNA-exposed animals (GUANG *et al.* 2008, 2010), First, we first asked whether the NRDE pathway and DAF-12 are required to silence the same cohort of genes. The NRDE pathway does not affect all RNAi, but selectively targets transcripts that are localized in nuclei (GUANG *et al.* 2008). These are mRNAs that may take longer to become spliced or to transit from the nucleus to the cytoplasm. We fed *daf-12* mutants and *nrde-3* mutants with various dsRNA-expressing food and compared the severity of their RNAi phenotypes with that appearing in wildtype animals (Table 2.2). Both *daf-12* and *nrde-3* are required for strong response to *pha-4* RNAi, *lin-26* RNAi, and *par-1* RNAi (Table 2.2, Fig. 2.19 and Fig. 2.20) (ZHUANG *et al.* 2013), suggesting that DAF-12 and NRDE-3 are required to silence these genes robustly. Of note, the degree of the NRDE-3 effect was significantly greater than that of DAF-12. For instance, when *pha-4* RNAi was strong enough to cause 100% lethality in wildtype embryos, 82.65% of *nrde-3* mutants were viable, whereas the viability of *daf-12* mutants was only 3.25%. On the other hand, when fed with bacteria expressing *skn-1*, *pos-1*, or *pal-1* dsRNA, neither DAF-12 nor NRDE-3 altered the efficiency of silencing

Table 2.2. RNAi phenotypes of some genes are enhanced or repressed in *daf-12* and *nrde-3* mutants.

Wildtype, *daf-12* mutants, and *nrde-3* mutants were fed with dsRNA targeting genes listed below. Repression (Rep)/enhancement (Enh) indicate equal or greater than 1.5 fold change in RNAi phenotype of the mutants compared to wildtype. NT, Not tested. NSD, No significant difference. *Tested by (ZHUANG *et al.* 2013). **Tested by (GUANG *et al.* 2008).

RNAi target gene					RNAi phenotype compared to wildtype	
Name	Identity	Role	Nuclear lamina enrichment	RNAi phenotype	*daf-12* (rh61rh411)	*nrde-3* (gg066)
pha-4	FoxA transcription factor	Pharynx development. Diet-restriction induced longevity	High	Emb. lethality	Rep	Rep
lin-26	Zinc-finger protein	Differentiation of non-neuronal ectodermal cells and the somatic gonad epithelium	Low	Emb. lethality	Rep	Rep
par-1	serine-threonine kinase	Embryonic polarity	Low	Emb. lethality	Rep	Rep*
F33H2.5	Unknown	Function unknown. Maternal effect gene	High	Emb. lethality	Rep	NT
mex-3	RNA binding protein	AB and P3 blastomere specification	High	Emb. lethality	Enh	NT
skn-1	bZip transcription factor	EMS blastomere specification.	Low	Emb. lethality	NSD	NSD

Table 2.2 (continued). RNAi phenotypes of some genes are enhanced or repressed in *daf-12* and *nrde-3* mutants

RNAi target gene					RNAi phenotype compared to wildtype	
Name	Identity	Role	Nuclear lamina enrichment	RNAi phenotype	*daf-12* (rh61rh411)	*nrde-3* (gg066)
pal-1	Homeodomain protein	Specification of posterior blastomeres	Low	Emb. lethality	NSD	NSD
pos-1	CCCH-type zinc-finger protein	Specification of germ cells, intestine, pharynx and hypodermis	Low	Emb. lethality	NSD	NSD**
glp-1	Notch receptor	Specification of germline and somatic tissues	Low	Emb. lethality	NSD	NT
elt-2	GATA-type transcription factor	Intestine development	Low	Emb. lethality	NSD	NT

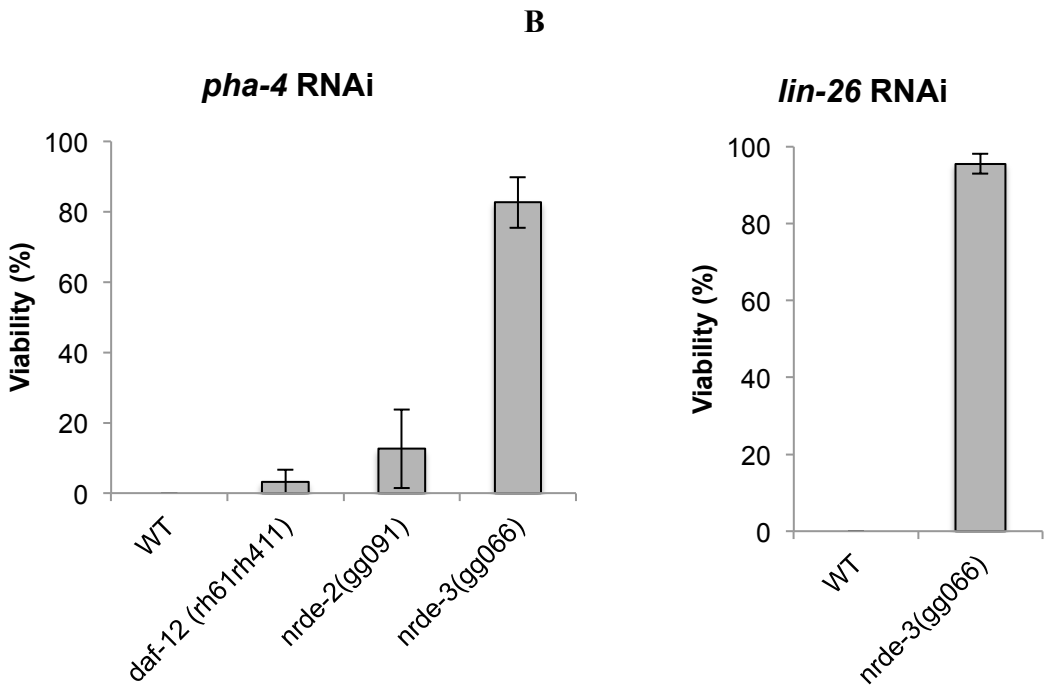
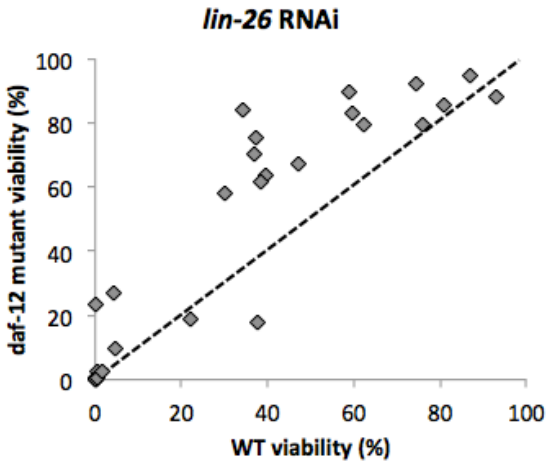


Figure 2.19. NRDE-3, a nuclear argonaute, is required for *pha-4* RNAi and *lin-26* RNAi.

Both *pha-4* RNAi (A) and *lin-26* RNAi (B) cause lethality. Animals were treated with *pha-4* and *lin-26* RNAi and the number of their progeny that grew to adulthood was scored. Although NRDE-2 interacts with NRDE-3 and act in the same pathway, its effect in RNAi is smaller than that of NRDE-3. The difference in RNAi sensitivity between *nrde-2* and *nrde-3* mutants has been reported in other independent study (ZHUANG *et al.* 2013). n=3. Error bars represent standard deviation.

A



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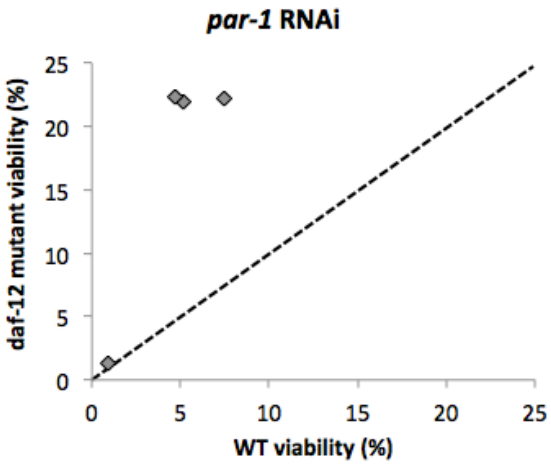


Figure 2.20. DAF-12 is required for robust *lin-26* RNAi and *par-1* RNAi.

lin-26 RNAi

(A) and *par-1* RNAi (B) lead to lethality. The dotted lines with the slope of one are superimposed to the graphs to aid the comparison between the viabilities of wildtype (WT) and *daf-12*(rh61rh411) mutants. Wilcoxon Signed-Rank test indicates $p<0.05$ for both *lin-26* RNAi and *par-1* RNAi. n=7 for *lin-26* RNAi; n=4 for *par-1* RNAi.

Table 2.2, Fig. 2.21 and Fig. 2.22). The overlap between the lists of genes that required DAF-12 or NRDE-3 for silencing raises the possibility that DAF-12 and NRDE-3 act in a common pathway. It is also plausible that DAF-12 functions in parallel to the NRDE pathway to silence the same cohort of genes.

DAF-12 acts as a transcription factor and the transcripts of the crucial components in the NRDE pathway— *nrde-1*, *nrde-2*, *nrde-3*, and *nrde-4*—are present in early embryos (LEVIN *et al.* 2012). Therefore, we hypothesized that DAF-12 might activate the expression of genes in the NRDE pathway in mothers, leading to a large maternal load and strong silencing in progeny. To test this idea, we collected early embryos and performed RT-qPCR for NRDE pathway genes. Quantitative PCR analysis using F35G12.2 as a reference gene (ZHANG *et al.* 2012) revealed that the transcript levels of *nrde-1*, *nrde-2*, *nrde-3*, and *nrde-4* in *daf-12* mutants were not significantly different from those in wildtype (Fig. 2.23A). We also examined the mRNA level of *hrde*-1, which encodes a germline Argonaute protein involved in the inheritance of RNAi (BUCKLEY *et al.* 2012) and found that DAF-12 did not affect the transcription of *hrde-1* (Fig. 2.23A). These results suggest that active transcription of the gene targeted by RNAi in *daf-12* mutants is not explained by the low expression of the NRDE factors.

To address the possibility that DAF-12 modulates some other factors that influence the activity of the NRDE pathway, we examined the mRNA levels of genes targeted by endogenous small interfering RNA (endo-siRNAs) in early embryos. Previous studies have shown that the argonautes in the NRDE pathway—NRDE-3 and HRDE-1—are associated with endo-siRNAs and facilitate silencing of genes targeted by endo-siRNAs (GUANG *et al.* 2008; BURKHART *et al.* 2011; ZHUANG *et al.* 2013).

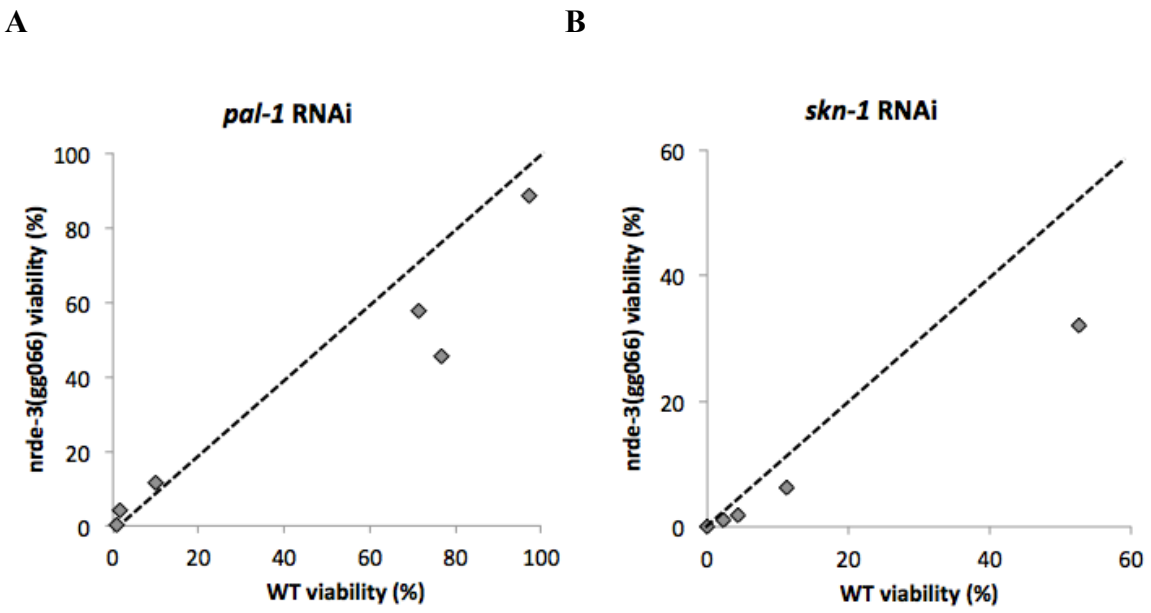
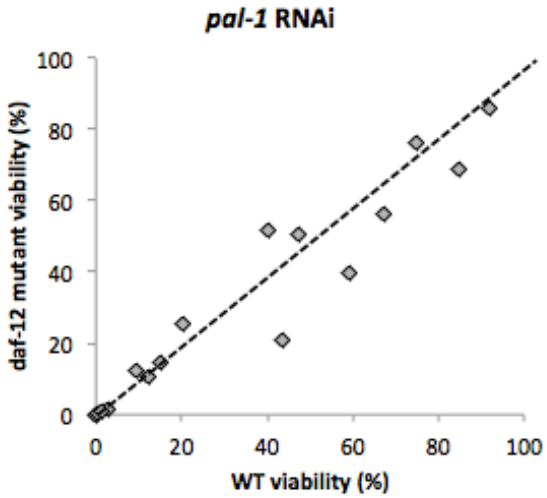


Figure 2.21. NRDE-3 is not required for *pal-1* RNAi and *skn-1* RNAi.

pal-1 RNAi (A) and *skn-1* RNAi (B) cause embryonic lethality. Wildtype (WT) and *nrde-3*(gg066) mutants were treated with *pal-1* and *skn-1* RNAi and the number of their progeny that grew to adulthood was scored. The dotted lines with the slope of one were drawn to better determine whether the viabilities of wildtype and *nrde-3*(gg066) mutants are similar or different. Wilcoxon Signed-Rank test returned $p>0.05$ for *pal-1* RNAi. The *p* value for *skn-1* RNAi was smaller than 0.05, but the fold change was not greater than 1.5. n=4 for both *pal-1* RNAi and *skn-1* RNAi.

Figure 2.22. DAF-12 is not required for *pal-1* RNAi, *pos-1* RNAi and *skn-1* RNAi. *pal-1* RNAi (A), *pos-1* RNAi (B), and *skn-1* RNAi (C) cause embryonic lethality. The dotted lines with the slope of one were drawn to better determine whether the viabilities of wildtype and *daf-12*(rh61rh411) mutants are similar or different. Wilcoxon Signed-Rank test shows $p>0.05$ for *pal-1* RNAi and *skn-1* RNAi. The p value for *pos-1* RNAi is greater than 0.05, but the fold change is smaller than 1.5. n=6 (*pal-1* RNAi), 8 (*pos-1* RNAi), and 7 (*skn-1* RNAi).

A



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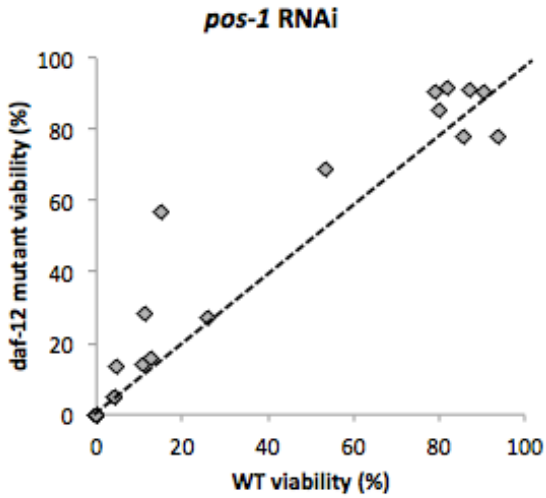


C

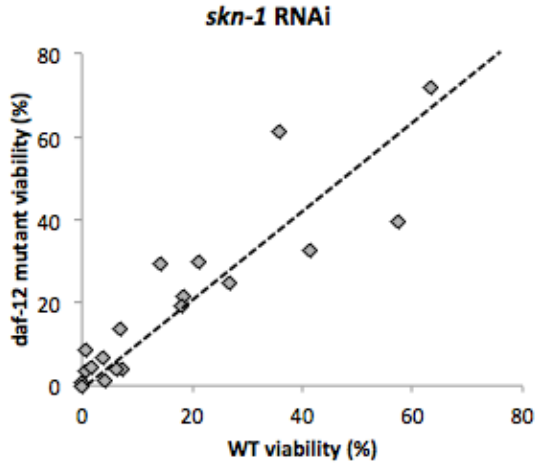


Figure 2.22 (continued). DAF-12 is not required for *pal-1* RNAi, *pos-1* RNAi and *skn-1* RNAi.

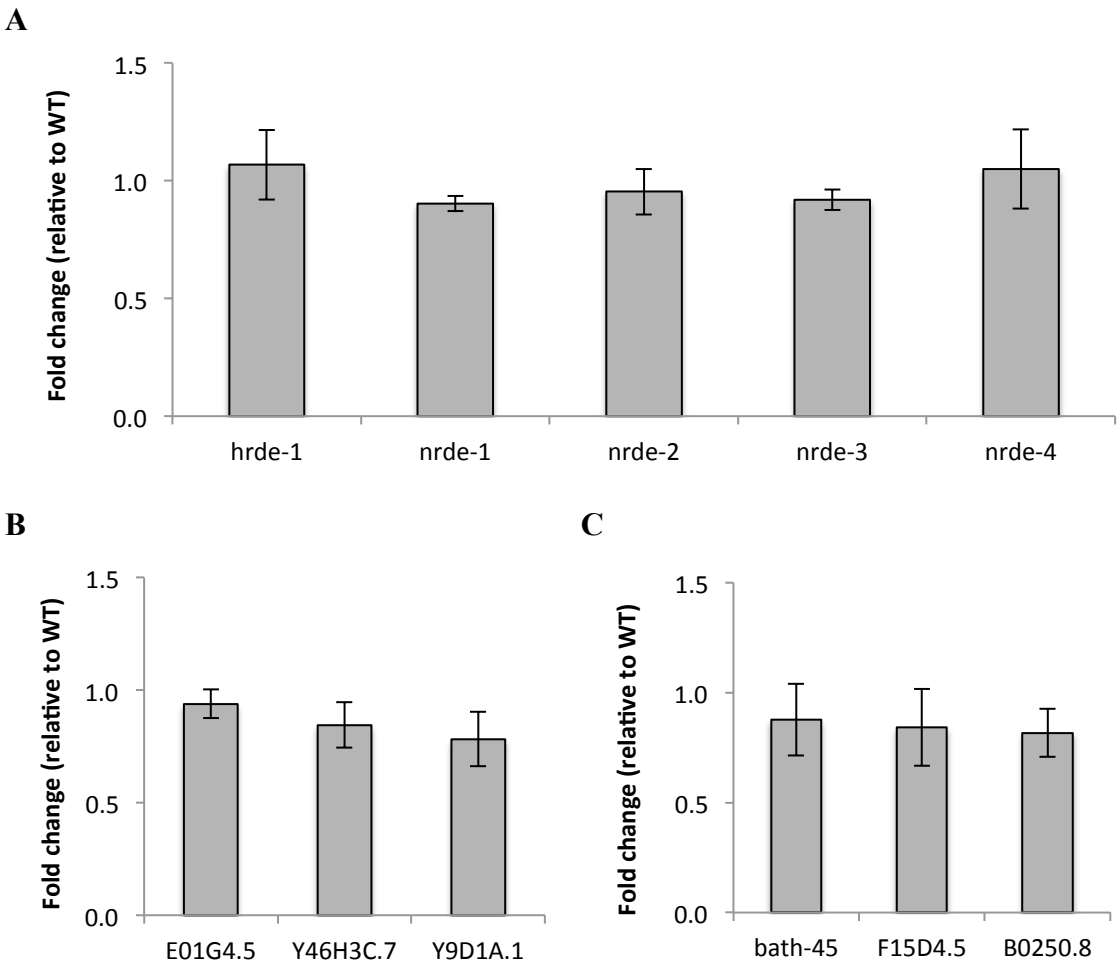


Figure 2.23. The activity of the NRDE pathway is not altered in *daf-12* mutant embryos.

Total RNAs from wildtype (WT) and *daf-12*(rh61rh411) mutant embryos were subjected to the RT-qPCR analysis. The 2-ΔΔCt method (LIVAK and SCHMITTGEN 2001) was used to compare the abundance of specific transcripts in *daf-12* mutants to that in wildtype. The value of one indicates the same level of the transcript in wildtype and *daf-12* mutant embryos. n=3. Error bars represent standard deviation. (A) Genes encoding the NRDE pathway components. (B) Genes regulated by the NRDE-siRNA pathway. (C) Genes regulated by the HRDE-siRNA pathway.

E01G4.5, Y46H3C.7, and Y9D1A.1 are among the genes targeted by NRDE-3-interacting endo-siRNAs (GUANG *et al.* 2008; BURKHART *et al.* 2011). Lack of NRDE-3 increases the mRNA level of E01G4.5 four fold (GUANG *et al.* 2008). In addition, Y46H3C.7, and Y9D1A.1 are highly marked with H3K9me3 in wildtype, but less enriched with H3K9me3 in NRDE pathway mutants (BURKHART *et al.* 2011), indicating that transcription of these endo-siRNA target genes are under the control of the NRDE pathway. We surveyed these genes to determine the level of activity of the NRDE pathway in *daf-12* mutants. Our RT-qPCR analysis revealed that early *daf-12* mutant embryos showed no difference in the transcript levels of E01G4.5, Y46H3C.7, and Y9D1A.1 compared to wildtype (Fig. 2.23B). The HRDE-1 activity seems unchanged in *daf-12* mutant embryos as well since *bath-45, F15D4.5,* and *B0250.*8, which are targeted by endo-siRNAs associated with HRDE-1 and up-regulated four fold in *hrde-1* mutants (BUCKLEY *et al.* 2012), were transcribed normally in *daf-12* mutant embryos (Fig. 2.23C). In summary, DAF-12 does not significantly change the activity of the NRDE pathway and may therefore act in parallel to the NRDE pathway to ensure transcriptional repression in response to RNAi.

Discussion

Here we have identified the *C. elegans* nuclear hormone receptor DAF-12 as a novel RNAi regulator. In *C. elegans*, RNAi-induced silencing activity persists in the progeny of dsRNA-exposed animals (FIRE *et al.* 1998; GRISHOK *et al.* 2000; VASTENHOUW *et al.* 2006; ALCAZAR *et al.* 2008). Recent studies have revealed that the inheritance of RNAi is associated with the presence of siRNAs and H3K9me3 at the locus targeted by RNAi over multiple generations (GUANG *et al.* 2010; BURTON *et al.* 2011; BUCKLEY *et al.* 2012;

GU *et al.* 2012). However, how the silencing response is passed down to and re-established in progeny is not well understood. In this study, we found that DAF-12 normally acts in mothers to increase the robustness of RNAi in zygotes. When DAF-12 is functional, RNAi-induced silencing is associated with low Pol II occupancy at the *pha-4* target locus, whereas lack of DAF-12 led to an increase in Pol II enrichment, although we recognize that post-transcriptional mechanisms may also contribute to the DAF-12 effects.

The role of DAF-12 in RNAi enhancement depends on the availability of DAF-12 ligands and DAF-12 co-repressors. Based on previous studies, which suggested that DAF-12 integrates multiple pathways responding to environmental cues, we tested and found that the environmental condition during mother's development determines the effect of DAF-12 on RNAi.

DAF-12 as a novel RNAi regulator

Almost all factors that are known to alter RNAi efficiency are intrinsic to the RNAi process. For instance, a mutation in *rde-4*, which encodes a dsRNA-binding protein that promotes siRNA production (PARRISH and FIRE 2001) confers a strong RNAi-defective phenotype (TABARA *et al.* 1999), whereas worms lacking functional ERGO-1, an argonaute involved in endogenous RNAi, show an enhanced RNAi phenotype (YIGIT *et al.* 2006; ZHUANG and HUNTER 2011). Unlike these factors, DAF-12 does not have any RNA binding domain (ANTEBI *et al.* 2000). Then, how could a nuclear hormone receptor increase RNAi efficiency? Considering its identity as a transcription factor, it is possible that DAF-12 activates expression of some unknown factors in the RNAi mechanism.

In addition to the molecular function, the mode of action of DAF-12 in RNAi is unique. *pha-4* RNAi treatment decreased Pol II occupancy at the *pha-4* locus in wildtype, whereas high enrichment of Pol II was detected in the *pha-4* gene body in *daf-12* mutants (Fig. 2.10D and E). This pattern was distinct from that of one of the NRDE pathway mutants, where Pol II levels at the targeted locus did not change upon RNAi (GUANG *et al.* 2010). Moreover, RNAi-triggered H3K9me3 modification still appeared in *daf-12* mutants after *pha-4* RNAi, whereas a NRDE pathway mutant fails to accumulate H3K9me3 at the targeted locus. These results indicate that the mechanism by which DAF-12 regulates RNAi is distinct from that of the NRDE pathway.

The contribution of DAF-12 to cross-generational signaling by RNAi

Two observations suggest that DAF-12 facilitates the inheritance of RNAi, rather than promoting the efficiency of RNAi in general. First, *daf-12* heterozygous mutants from homozygous mothers failed to show the robust RNAi response, indicating that DAF-12 is required in mothers to enhance RNAi efficiency in zygotes. Second, *daf-12* mutants (P0) that were directly exposed to RNAi were as responsive to RNAi as wildtype animals: we observed weak *pha-4* RNAi phenotypes only in the F1 generation. This characteristic distinguishes DAF-12 from other factors—such as RDE-1 and RDE-4—which, when mutated, confer defects in the RNAi process in both dsRNA-treated animals and their progeny (TABARA *et al.* 1999; PARRISH and FIRE 2001).

How could DAF-12 regulate silencing induced in the inheriting zygotes? The RNAi process generates two classes of siRNAs —primary and secondary. Primary siRNAs are derived from the initial dsRNA by the activity of Dicer (ELBASHIR *et al.*

2001; BERNSTEIN *et al.* 2001). When primary siRNAs bind to their target transcripts, they can act as primers for RNA-dependent RNA polymerase (RdRP) and as a consequence, secondary siRNAs are produced (SMARDON *et al.* 2000; AOKI *et al.* 2007; GHILDIYAL and ZAMORE 2009). A previous study showed that both primary and secondary siRNAs are inherited in the progeny (F1) of the animals (P0) directly exposed to dsRNA, although the levels of primary and/or secondary siRNAs were much lower in the offspring than in the P0 animals (GU *et al.* 2012). It is possible that DAF-12 increases the number of primary or secondary siRNAs deposited into oocytes. *daf-12* is expressed in multiple tissues including the intestine and gonad (ANTEBI *et al.* 2000), but its expression has not been detected in the germ line or early embryo (BAUGH *et al.* 2003; REINKE *et al.* 2003; YUZYUK *et al.* 2009; LEVIN *et al.* 2012). Intestinal cells need to transport ingested dsRNAs or processed siRNAs to the gonad and to the germ line for inheritance (JOSE and HUNTER 2007). Therefore, one speculative idea is that DAF-12 facilitates the inter-cellular transport of silencing signals to the germ line. Another possibility is that maternal DAF-12 up-regulates the activity of the RNAi process in zygotes to re-establish the silenced state. We found that the expression of the NRDE pathway components and their activity were not altered in *daf-12* mutants (Fig. 2.23A). Therefore, DAF-12 may regulate an unknown factor or process in parallel to the NRDE pathway. If this scenario is the case, how *daf-12* expressed in somatic cells of mothers can exert an effect in zygotes remains to be seen.

The environmental effect on RNAi

One of the intriguing findings in our study was that high population density during mothers' larval development restored a strong RNAi phenotype to *daf-12* mutants, which

had shown a weak RNAi phenotype when cultured in an under-crowded condition (Fig. 2.15B). In contrast, the strength of the RNAi phenotype was strong in wildtype animals regardless of the maternal population density during development (Fig. 2.15B). These results suggest that under-crowded conditions can possibly strengthen RNAi response, but that DAF-12 mitigates this effect. A previous study proposed that DAF-12 acts as a capacitor (or a buffer); environmental fluctuations can be potentially harmful and cause phenotypic changes, but DAF-12 minimizes such effect (HOCHBAUM *et al.* 2011). Our work reinforces the role of DAF-12 as a buffer to alleviate any environmental effect on physiological changes.

Previous studies reported that RNA-defective (Rde) phenotypes in some mutants are dependent on temperature (HABIG *et al.* 2008; HAN *et al.* 2008). In these studies, animals were maintained at a certain temperature for the whole period of RNAi. On the contrary, in our experimental setup, crowding versus sparse condition was limited to the larval growth of mothers. How could animals "remember" their previous environment and alter molecular/physiological phenotypes in embryos? Recently, studies have amassed examples where transient experience of certain environmental factors, such as pollutants (ANWAY *et al.* 2005; MANIKKAM *et al.* 2012; DOYLE *et al.* 2013) and particular diets (CARONE *et al.* 2010; NG *et al.* 2010; FULLSTON *et al.* 2013), in one generation could alter the phenotypes of the subsequent generation. For example, when male P0 mice learned to link a certain odor (acetonphenone) with a noise that provoked a startle response, F1 and F2 offspring showed a high sensitivity to the odor, although they were never conditioned (DIAS and RESSLER 2013). This work suggests that the memory of the exposure could be transmitted across generations. Our study not only provides another

example where the memory of an environmental condition long before mating is transmitted to zygotes and influences their phenotypes, but also suggests a potential interaction between environment and small RNA pathways.

CHAPTER III

Summary and Future Directions

Summary

RNA interference (RNAi) is a cellular process where small non-coding RNAs target and silence the expression of the complementary transcripts by transcriptional and post-transcriptional mechanisms (QUE and JORGENSEN 1998; FIRE *et al.* 1998; MONTGOMERY *et al.* 1998; GUANG *et al.* 2008, 2010). In *C. elegans*, ingested dsRNA can trigger RNAi in animals directly exposed to dsRNA and elicit a strong RNAi response in their progeny, in which target mRNAs are degraded in response to complementary small RNAs (MONTGOMERY *et al.* 1998; TABARA *et al.* 1999; TUSCHL *et al.* 1999; HAMMOND *et al.* 2000). Extensive investigations uncovered that a significant level of small RNAs as well as the H3K9me3 modification persist in the progeny of dsRNA-exposed animals (GUANG *et al.* 2010; BURTON *et al.* 2011; GU *et al.* 2012), indicating that small RNAs and the chromatin state may be responsible for the transmission of RNAi across generations or are re-established de novo at each generation. A pathway to establish silencing in progeny of dsRNA-exposed animals is known, but a maternal contribution to RNAi inheritance has not been observed. Here we show that maternal DAF-12 is required for robust RNAi in zygotes. Our analyses showed that the DAF-12/nuclear hormone receptor is required in mothers to induce strong RNAi phenotypes in zygotes. Pol II ChIP-qPCR analysis revealed that DAF-12 enhances RNAi by inhibiting transcription of the targeted gene. We also found that the role of DAF-12 in RNAi regulation is independent of other

pathways that DAF-12 is involved. These findings implicate that animals exposed to dsRNAs are actively engaged in inducing RNAi response in zygotes and that DAF-12 provides additional layer of regulation in this process. In addition, we discovered that the effect of DAF-12 on RNAi was modulated by the environmental conditions during mothers' development, which suggests an intriguing link between the experience of environmental cues and RNAi inheritance. Together, the work presented here provides insights into cross-generational signaling that modulates RNAi-induced silencing (Fig. 3.1). Moreover, given the inter-dependent nature of multiple small RNA pathways that are involved in various biological mechanisms such as fertility and genome stability, it is plausible that the interaction between the environment and the RNAi mechanism may affect other biological processes.

Discussion

Translation of maternal experience into molecular changes in zygotes

One of the major findings of this study is the requirement of maternal DAF-12 for establishment of RNAi-triggered silencing activity in zygotes. Our understanding of RNAi inheritance has expended recently, but the identity of molecules that are transmitted from dsRNA-exposed mothers to their zygotes is still unknown. The identification of DAF-12 acting in mothers as a mediator of RNAi inheritance not only highlights a significant role of mother in passing on the silencing response to zygotes, but also provides a tool to hunt for the molecules carrying the memory of RNAi-induced silencing (see the Future Direction).

Figure 3.1 Models for the effects of environment and DAF-12 on RNAi efficiency. My work suggests that maternal DAF-12 is required for robust RNAi in zygotes. The observations that *daf-4*/TGF-beta receptor was epistatic to *daf-12* and that DAF-12 became dispensable when worms developed in a crowded condition indicate that DAF-12 acts either upstream of a neuronal signaling and the TGF-beta pathways (A) or in parallel to the TGF-beta pathway responding to environmental cues (B). These two possibilities can be differentiated by the further analysis on the genetic interactions between *daf-12* and the components required for the neuronal signaling and the TGF-beta pathways. How the effect of DAF-12 on RNAi in zygotes is mediated remains to be seen. ChIP-qPCR analysis revealed that DAF-12 promotes a decrease in Pol II occupancy at the *pha-4* locus upon RNAi, but the RNAi-triggered accumulation of H3K9me3 is not dependent on DAF-12. This specific effect makes a contrast to the NRDE pathway, which both inhibits the transcription elongation step and increases the level of H3K9me3 at the locus targeted by RNAi (GUANG *et al.* 2010). Thus, we conclude that DAF-12 may regulate RNAi independently of the NRDE pathway. It is possible that the DAF-12—DIN-1 repressor complex inhibits expression of a gene or genes in the germ line that weaken the RNAi-induced silencing activity in early embryos. Or DAF-12 may facilitate transport of small RNAs derived from the initial dsRNAs into gonad and germ cells to provide a sufficient amount of small RNAs to trigger silencing activity in zygotes. Another possibility is that an unknown chromatin mark associated with RNAi-induced silencing is inherited to the progeny in a DAF-12- dependent manner.

A. **B.**

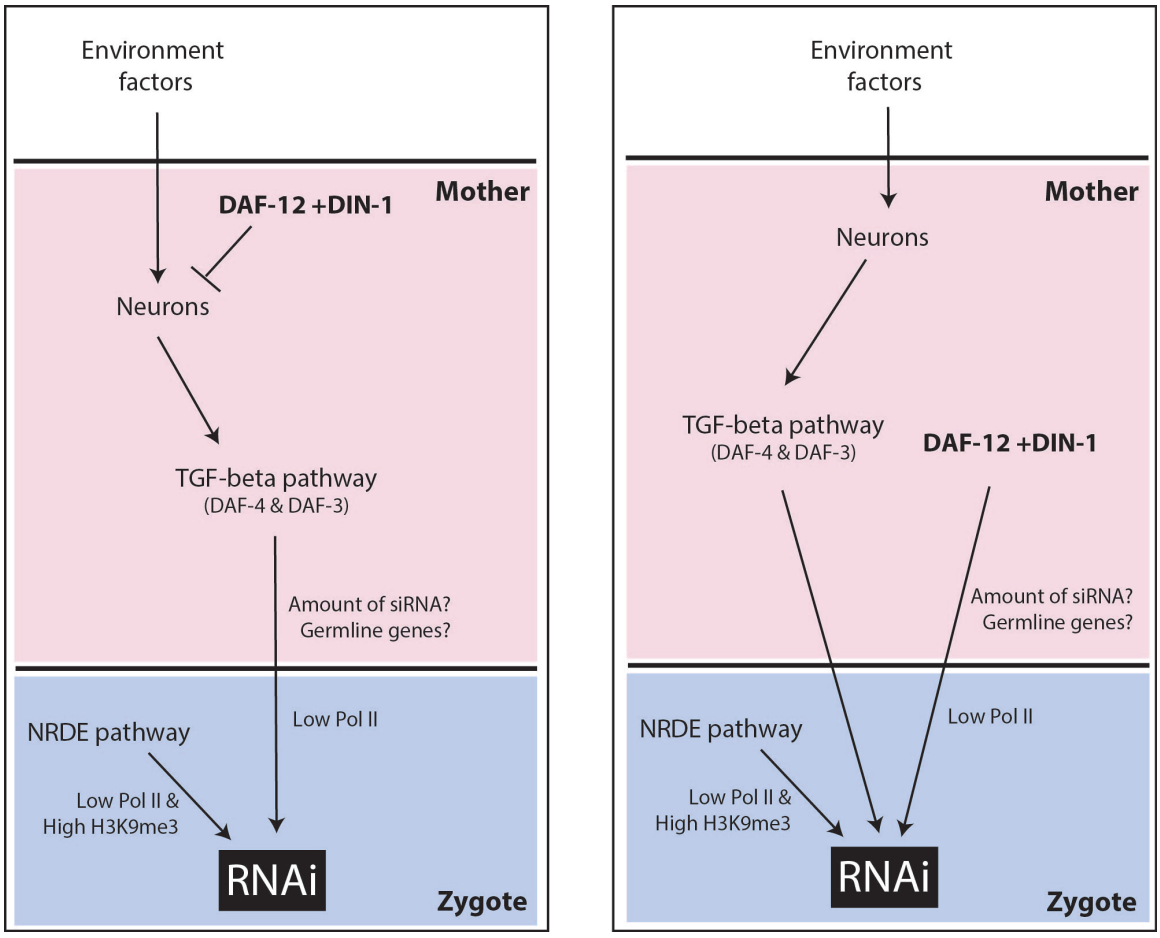


Figure 3.1 (continued). Models for the effects of environment and DAF-12 on RNAi efficiency.

In addition, we found that *daf-12* mutants, which normally show a weak RNAi response, exhibited robust RNAi when their mothers were reared under the crowded environment (Fig. 2.15B). Exposure to crowded environment was limited to during mothers' development, indicating a long-lasting effect of transient maternal experience on the phenotype of zygotes. How a memory of experiences could be passed down to the future generation remains to be seen, but our finding provides an example, where environmental conditions leave a cross-generational impact.

It is of note that wildtype animals showed no difference in RNAi efficiency upon different environmental settings (Fig. 2.15B). How could the environmental conditions have an impact only in *daf-12* mutants? A plausible explanation is that functional DAF-12 in wildtype animals acts as a buffer to minimize the effect of environmental changes. The role of DAF-12 as a capacitor has been suggested previously, and the similar concept could be applied here. Another speculative model is that the requirement of DAF-12 for robust RNAi depends on environmental conditions; DAF-12 modulates RNAi efficiency only when mothers grow in a less-populated condition. A high population density, on the other hand, activates some other components regulating RNAi and DAF-12 is no longer needed for robust RNAi. This model explains why we saw the effect of DAF-12 only when worms were sparse.

RNAi-induced H3K9me3 enrichment

One signature of transcriptional repression induced by RNAi is the enrichment of H3K9me3 (BURTON *et al.* 2011; GU *et al.* 2012), Our H3K9me3 ChIP revealed that both wildtype and *daf-12* mutants show an increase in the H3K9me3 level at the region

targeted by the initial trigger dsRNA (Fig. 2.11). One caveat of these experiments is the mixture of cell types that are monitored. Normally, gut cells express PHA-4 whereas other types of cells do not (AZZARIA *et al.* 1996; KALB *et al.* 1998). Subjecting whole embryos to ChIP might prevent us from recognizing a difference in the foregut-specific H3K9me3 enrichment upon *pha-4* RNAi. Presumably, the *pha-4* locus in non-pharyngeal cells, which make up the majority of cells in an embryo, is transcriptionally inactive, and likely marked with H3K9me3. Therefore, any slight but significant change in H3K9me3 at the *pha-4* locus in pharyngeal cells may not be detectable when both pharyngeal and non-pharyngeal cells were subjected to ChIP. Nevertheless, the increase in H3K9me3 reveals that changes in response to RNAi were detectable in this experiment.

Transcription inactivation triggered by pha-4 RNAi

Kennedy and colleagues reported that the elongation step of transcription was inhibited by the NRDE pathway during RNAi (GUANG *et al.* 2010). Comparing the Pol II occupancy with or without *lin-15b* RNAi treatment, the authors observed no change in Pol II occupancy near the start of the first exon. However, there was a two-fold increase in Pol II enrichment at the region targeted by dsRNA and a dramatic reduction of Pol II occupancy downstream of the targeted region. The Pol II level at the 3' end of the coding sequences was half that of samples without RNAi treatment. This Pol II pattern of accumulation and decrease was dependent on NRDE-2, an essential nuclear component of the NRDE pathway (GUANG *et al.* 2010). Similarly, Burkhart and colleagues used nuclear run-on assays, which capture nascent transcripts (CORE *et al.* 2008), to show a

reduction of transcription downstream of the site targeted by dsRNA (BURKHART *et al.* 2011).

The change in the Pol II enrichment pattern at the *pha-4* locus after *pha-4* RNAi was very different from what Kennedy and colleagues reported. Upon *pha-4* RNAi, Pol II occupancy dropped to half of its normal level at the start of coding sequence and remained low throughout the gene body (Fig. 2.10D). The discrepancy between the Pol II patterns that Guang *et al* (2010) reported and that we observed could reflect the different genes targeted, or differences in silencing between the two studies. For example, *pha-4* likely auto-regulates itself, so a drop in PHA-4 after RNAi may lead to a general drop in its own transcription. With either explanation, the observed changes in Pol II occupancy suggest that transcription initiation, not elongation, falls after *pha-4*(RNAi).

In *daf-12* mutants, the Pol II enrichment at the *pha-4* gene body increased two fold after RNAi (Fig. 2.10D and E). The difference in Pol II occupancy near the promoter region was insignificant, but the regions before and after the site targeted by the initial trigger dsRNA showed high Pol II enrichment (Fig. 2.10D and E). This trend seems different from that of stalled Pol II during the elongation step, which is characterized by a greater than four-fold decrease in the level of Pol II bound to DNA downstream of the region targeted by dsRNA (GUANG *et al.* 2010). Whether a two-fold increase in the Pol II enrichment at the gene body reflects more transcription needs to be determined, but the opposite directions of the Pol II accumulation in wildtype embryos versus *daf-12* mutants (a decrease in wildtype and an increase in *daf-12* mutants) raises the possibility that DAF-12 mediates responses to RNAi at the transcription level, either directly or indirectly.

What could explain RNAi-triggered accumulation of Pol II in *daf-12* mutants? A speculative model is that there is a mechanism that promotes transcription in response to RNAi, but this mechanism is inhibited by DAF-12 in embryos. The notion that dsRNA treatment can elicit transcriptional activation seems counter-intuitive, as exposure to dsRNA is known to reduce the expression level of the targeted gene. However, recent studies have identified an "anti-silencing" mechanism that involves the same type of 22G siRNAs that can also trigger silencing, depending on the proteins bound to the siRNA (Fig. 1.2) (CONINE *et al.* 2013; SETH *et al.* 2013; WEDELES *et al.* 2013). The dual role of siRNAs in germline gene expression/silencing implies that there may be a similar interaction between small RNA pathways for exogenous RNAi. Such "anti-silencing" pathway for exogenous RNAi and its interaction with DAF-12 remain enigmatic at this moment, but it would be an interesting future direction.

Distinct roles of DAF-12 in RNAi enhancement and developmental timing pathways

Our mutant analysis indicates that DAF-12 regulates RNAi via a novel pathway that is distinct from dauer formation and heterochronic pathways. We examined *pha-4* RNAi efficiency of multiple *daf-12* mutants that exhibit different dauer phenotypes and varying degrees of severity in heterochronic phenotypes. A wide range of phenotypes among *daf-12* mutants is attributed to the positions and the natures of mutations in *daf-12*, which are likely to alter its affinity to the ligand or the co-repressor DIN-1. The resulting changes in the ratio of unliganded DAF-12 and liganded DAF-12 may confer varying degrees of mutant phenotypes. However, the weak *pha-4* RNAi phenotype was universal among *daf-12* mutants tested in the study. This result raises the possibility that even a subtle

deviation from the normal ratio of unliganded DAF-12 to liganded DAF-12 may alter the efficiency of *pha-4* RNAi. Our analysis with *nhr-8* and *din-1* mutants further suggests that an increase in the DAF-12—DIN-1 repressor complex and a (reciprocal) reduction of liganded DAF-12 enhance silencing induced by RNAi. These findings suggest that, like other pathways in which DAF-12 is involved, the role of DAF-12 in RNAi also relies on the availability of its ligands, Dafachronic acids. How could one input—biosynthesis of DA and its binding to DAF-12—elicit three different responses? One possibility is that different thresholds of the ratio of unliganded DAF-12 to liganded DAF-12 are required for a certain phenotype to appear. For instance, the enhanced RNAi phenotype might require a slight increase in the level of DAF-12—DIN-1 complex, whereas the dauer phenotype might depend on a high concentration of DAF-12—DIN-1 complex. Alternatively, our analysis with multiple *daf-12* mutants demonstrates the importance of the fine-tuning process of the DAF-12 function in physiological changes.

The effect of dsRNA dosage on the role of DAF-12 in RNAi

Through serial dilution of RNAi food with neutral RNAi bacteria, we noticed that the effect of DAF-12 on RNAi enhancement was greatest when enough dsRNA was supplied for killing >50%. As dsRNA was diluted, *daf-12* mutants no longer showed a significant decrease in RNAi-induced lethality. This observation suggests that the amount of dsRNA should be above a certain level to trigger DAF-12-mediated RNAi inheritance. Further investigation in the mechanism by which DAF-12 enhances RNAi inheritance may explain the existence of threshold for DAF-12 to function.

Future Directions

Mechanisms by which maternal DAF-12 promotes RNAi in zygotes

The requirement of DAF-12 in mothers for robust RNAi in zygotes leaves a question of how this non-conventional maternal effect occurs. To answer this question, it would be informative to know when and where *daf-12* needs to be expressed in mothers to enhance RNAi in zygotes. *daf-12* is expressed in multiple tissues, including some neurons, pharynx, and intestine (ANTEBI *et al.* 2000). Tissue-specific expression of *daf-12* would be a good method, except that one should be cautious about using transgenes. We have encountered the issues where transgenic worms carrying transgenes are insensitive to *pha-4* RNAi (see Appendix). Use of single copy transgenes could solve this problem and aid the identification of the critical tissue(s) for DAF-12 function. Narrowing down the developmental time window, when DAF-12 is required, could be challenging. However, a tool recently developed by Judkins and his colleagues may help researchers have a tight temporal control of DA availability (JUDKINS *et al.* 2014). An experimental scheme would include 1) feeding *daf-9* mutant worms, which cannot synthesize DA, with photocleavable amides of 5-methoxy-N-methyl-2-nitroaniline (MMNA)-protected DA; 2) exposing worms to UV irradiation at various time points during larval development; 3) performing *pha-4* RNAi and examining RNAi phenotypes in offspring. Using this technique, one could pinpoint a specific developmental period when DAF-12 influences RNAi inheritance. Identification of the spatial and temporal requirements of DAF-12 for RNAi enhancement would help us to better understand how parents exposed to dsRNA can ensure the robust RNAi in zygotes.

In the current study, we examined the effect of DAF-12 on RNAi targeting genes functioning during embryogenesis. An interesting future question is whether DAF-12 can enhance RNAi targeting genes that are expressed later in development. For instance, it was proposed that NRDE-3 is required for silencing genes that function during larval development (BURTON *et al.* 2011). Given our experiments, which showed that NRDE-3 is required for robust *pha-4* RNAi and *lin-26* RNAi, both of which cause embryonic lethality, the NRDE pathway is required for heritable silencing of genes expressed during embryogenesis. Experiments, where wildtype and *daf-12* mutants are fed with dsRNA targeting genes required later in development, would answer the question of whether the DAF-12 effect on RNAi is limited to embryonic genes or not. In our preliminary experiments, *daf-12* mutants exhibited strong *dpy-11* RNAi phenotype, whereas previous studies reported that NRDE-3 is required for robust *dpy-11* RNAi (GUANG *et al.* 2008; ZHUANG *et al.* 2013). More experiments with multiple RNAi would be required to obtain a solid conclusion. Of note, silencing genes that are expressed during larval stages might cause a range of phenotypes, not the binary, life or death, choices that we saw for *pha-4* and *lin-26*. For instance, a small change in Unc (uncoordinated) or Dpy (dumpy) phenotypes could be hard to discern. Therefore, meticulous observation and quantitation would be required to spot any significant effect on the efficiency of RNAi.

How could* daf-12 *mediate RNAi inheritance?

The current work presents an interesting cross-generational effect of DAF-12 on the RNAi efficiency. However, we do not know what mediates this effect. Any molecular signatures of RNAi—primary siRNA, secondary siRNA, and/or H3K9me3—could be the

critical effector of DAF-12. For instance, DAF-12 may change the amount of primary and/or secondary siRNAs deposited into oocytes. It is also possible that a zygotic factor that is regulated by DAF-12 in mothers plays a role in consolidating the silenced state of the gene targeted by RNAi. Analysis of the expression profiles of genes expressed in wildtype and *daf-12* germ lines and ChIP of DAF-12 protein (HOCHBAUM *et al.* 2011) may provide a clue of what factors mediate the DAF-12 effect. Most studies about DAF-12 have focused on its role in the processes occurring in one generation, such as larval development or longevity. Significant differences in the composition of maternally deposited factors between wildtype and *daf-12* have not been described but might reveal other candidates that are determine the potency of cross-generational signaling.

The contribution of the environment to RNAi

The environmental contribution to the DAF-12 effect on RNAi seems rather specific; cholesterol starvation decreased RNAi sensitivity in wildtype animals (See Appendix), whereas under-crowding versus over-crowding did not alter the RNAi phenotypes. Instead, the population density determined the influence of DAF-12 on RNAi enhancement. These results indicate that different environmental cues activate separate pathways to regulate RNAi efficiency. What pathways would be strong candidates to modulate RNAi in response to external conditions? Transduction signaling pathways that link certain environmental factors (i.e. chemicals or temperature) to physiological changes are well studied in *C. elegans*, compared to other model systems. We decided to look into TGF-beta dauer pathway due to its link with environmental inputs and DAF-12; it was known that a high concentration of a pheromone, which implies high population

density, inactivates the expression of TGF-beta ligand (REN *et al.* 1996; SCHACKWITZ *et al.* 1996), thus turning off the downstream pathway . Moreover, expression of the enzymes required for DA biosynthesis is determined by TGF-beta dauer pathway (JIA *et al.* 2002; GERISCH and ANTEBI 2004), indicating that this signaling pathway modulates the activity of DAF-12. In an attempt to test whether TGF-beta is involved in RNAi regulation, we tested TGF-beta pathway mutants with *pha-4* RNAi and discovered some candidate genes that may be involved in the *pha-4* RNAi regulation (Fig. 2.17B). We have not tested yet whether TGF-beta pathway mutants show weak RNAi in general or not. Therefore, it is not clear whether TGF-beta pathway influences RNAi efficiency or PHA-4 activity.

Among TGF-beta mutants, we chose worms carrying a deletion mutation in *daf-4*, which encodes TGF-beta receptor type II (ESTEVEZ *et al.* 1993), and looked at the genetic relationship between *daf-4* and *daf-12*. The epistasis analysis revealed that lack of DAF-12 did not alter RNAi efficiency in *daf-4* mutant background, just like crowded environmental condition (Fig. 2.18). The observation that both highly populated condition and a *daf-4* mutation, which confers constitutive dauer formation as if worms experience unfavorable environment, nullified the effect of DAF-12 on RNAi suggests the environmental effect on the robustness of RNAi.

An interesting future question to address is how environmental cues perceived by neurons could regulate the robustness of RNAi in the zygotes. Sensory neurons are located in the head of a worm, therefore they need a means to reach other tissues and ultimately germ line to modulate RNAi inheritance. Hormonal signals may facilitate such a long-distance communication between neurons and germ line. It has been shown that

ASI neurons in the head of an animal can communicate with the distal tip cells located in the gonad to regulate germ line development (DALFÓ *et al.* 2012). Together, identification of the sensory input, neurons, signaling transduction pathways connecting the neuronal activity and changes in germ line would help us put a complete picture of how environmental information in the parental generation could modulate the strength of RNAi in zygotes.

APPENDIX I

Non-genetic Factors That Regulate RNAi Efficiency

Introduction

Previous studies have identified numerous genes that can modulate exogenous RNAi activity. For instance, a mutation in *rde-4*, which encodes a dsRNA-binding protein (TABARA *et al.* 1999), confers a strong RNAi-defective phenotype, whereas worms lacking functional ERGO-1, an argonaute involved in endogenous RNAi, exhibit an enhanced RNAi phenotype (YIGIT *et al.* 2006; ZHUANG and HUNTER 2011). Although we have gained a comprehensive view of the RNAi process from these studies, we have little understanding of whether and what non-genetic, environmental factors alter the efficiency of RNAi. While studying the role of DAF-12 in RNAi inheritance, we identified some internal and external factors that change the efficiency of exogenous RNAi in zygotes. Brief descriptions of the methods and discussion are presented here. The underlying mechanisms remain to be determined.

Materials and Methods

Worm maintenance and *pha-4* RNAi

Worms were maintained and *pha-4* RNAi was done as described in Chapter 2

Ablation of ASI and ASK neurons

Animals were hatched and grown at 25°C. Worms at the L3 stage were washed off plates and incubated in 1ml of M9 containing 10mM of DiD (Invitrogen #904534) at 25°C for two hours with rocking. After incubation, worms were washed three times with M9 and transferred on a plate with OP50 food for 30 min. Three to five dye-filled L3 stage worms were immobilized on a 10% agar pad containing 20mM of sodium azide and covered with a coverslip. A pair of ASI, ASK, or ADL neurons was subjected to laser ablation. Once the fluorescent dye was no longer present in the cell body of the neurons, worms were washed off from the agar pad and recovered on an OP50-seeded plate at 25°C. Mock-ablated worms were treated the same way except laser firing was omitted. When worms reached the L4 stage, they were subjected to *pha-4* RNAi or *elt-2* RNAi as described above with one modification; instead of 15 worms, two or three L4 worms were placed per RNAi plate.

Results and Discussion

Cholesterol starvation in parents causes resistance to pha-4 RNAi in zygotes

C. elegans cannot synthesize cholesterol *de novo* (ROTHSTEIN 1968), thus dietary cholesterol is absolutely required for their development. Deprivation of cholesterol leads to defects in molting (YOCHEM *et al.* 1999) and facilitates the entry into the dauer stage (GERISCH *et al.* 2001). To determine whether cholesterol deprivation alters *pha-4* RNAi efficiency, we reared worms on cholesterol-deficient (vehicle only) plates from birth to the fourth larval stage and treated them with *pha-4* RNAi (Fig. 4.1). Of note, the worms

that grew without cholesterol underwent normal larval growth due to maternally deposited cholesterol and perhaps low levels of cholesterol in the media.

We found that the progeny of animals that were deprived of cholesterol during larval growth were four times more viable than those from animals that were supplemented with cholesterol (Fig. 4.2). This result indicates that *pha-4* RNAi was less effective when worms were grown without dietary cholesterol. This observation implies that a subtle change in cholesterol metabolism affects RNAi activity.

Dietary cholesterol is processed to become Dafachronic acids, which are the ligands of DAF-12 (MOTOLA *et al.* 2006; MAHANTI *et al.* 2014). Therefore, we hypothesized that dietary cholesterol would enhance RNAi via DAF-12 by promoting the unliganded version of DAF-12. However, like wildtype animals, *daf-12*(rh61rh411) null mutant embryos also showed less *pha-4*(RNAi)-associated lethality when their parents were grown without cholesterol (Fig. 4.2). This result suggests that the effect of dietary cholesterol on RNAi enhancement is independent of DAF-12.

There are two issues that should be resolved in future studies. First, it is possible that *pha-4* RNAi bacteria express less *pha-4* dsRNA when seeded on cholesterol-deficient plates and therefore worms show less severe RNAi phenotypes. To circumvent this issue, an alternative method of RNAi, such as soaking worms in a dsRNA solution or injecting dsRNAs into worms, could be used. Second, one needs to test RNAi against multiple genes to determine whether the effect of cholesterol on RNAi is general or specific to *pha-4*.

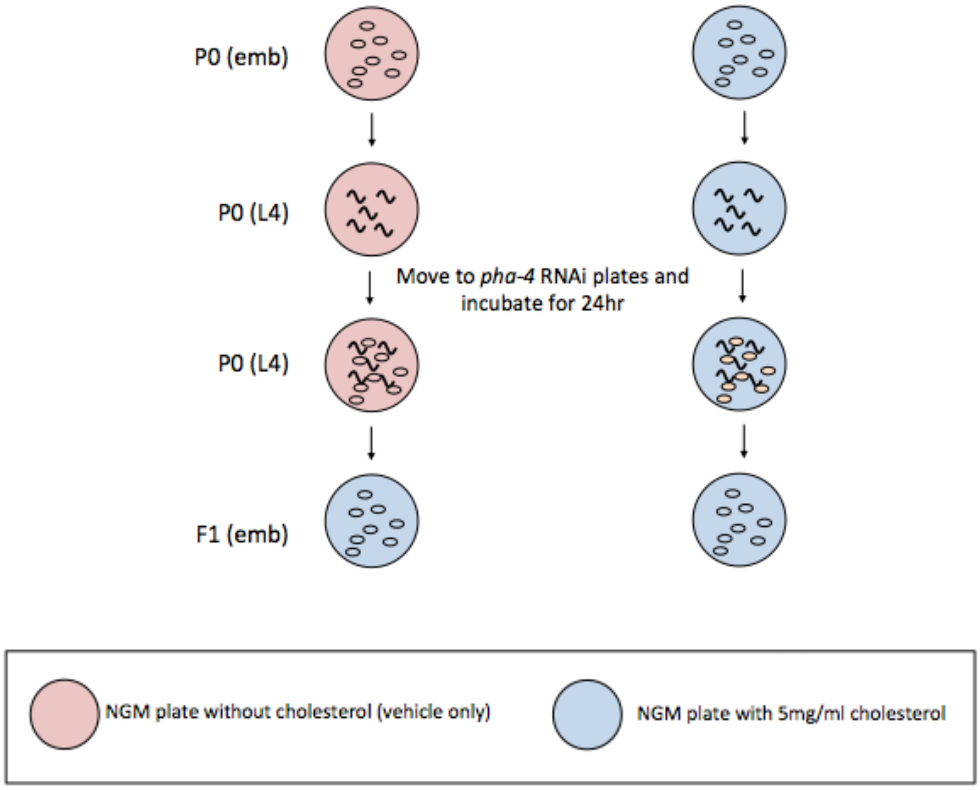


Figure 4.1. A schematic diagram of the experiment to test the role of dietary cholesterol in RNAi.

Worms (P0) were hatched and grew either without cholesterol (pink; ethanol—the solvent of cholesterol—was added to the plate) or with 5mg/ml of cholesterol (blue). Due to the maternally deposited cholesterol, the worms reared on the plate devoid of cholesterol (left; pink) underwent normal larval growth. When animals reached the fourth larval stage (L4), 15 worms from each treatment were transferred to the plates that were seeded with *pha-4* dsRNA-expressing bacteria. Later, the viability of the progeny (F1) was scored.

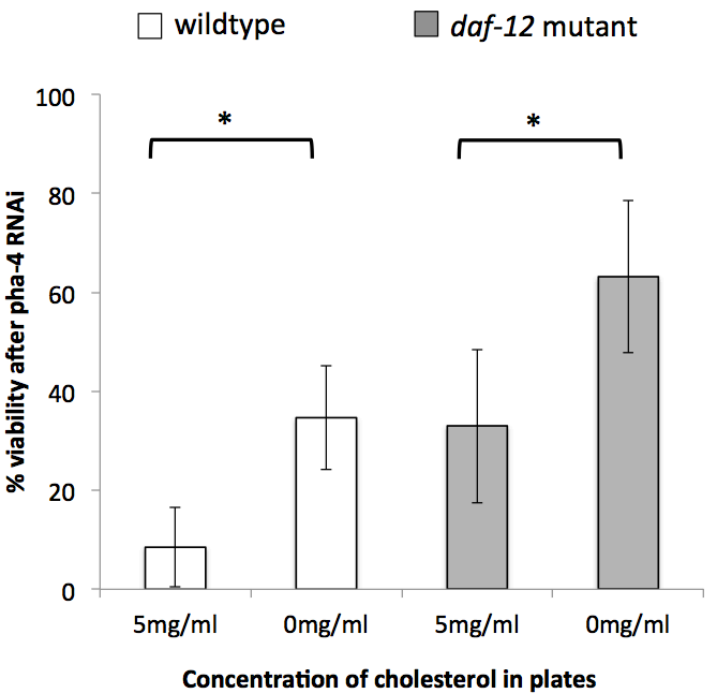


Figure 4.2. Cholesterol deprivation decreased the *pha-4* RNAi efficiency.

Animals were incubated with or without cholesterol throughout their larval development and exposed to *pha-4* RNAi when they were at the fourth (last) larval stage. The animals produced progeny that exhibited different levels of *pha-4* (RNAi)-associated lethality. n=3. * $p<0.05$ (Student t-test).

Extra-chromosomal arrays prevent strong response to pha-4 RNAi.

When injected with mixtures of DNA molecules, *C. elegans* has the ability to form an array that can propagate through mitosis and meiosis (MELLO *et al.* 1991). This extra-chromosomal array is typically composed of repetitive sequences (thus high-copy number) of the injected DNA molecules and sometimes subjected to transcriptional repression (MARTIN and WHITELAW 1996; HSIEH and FIRE 2000). High-copy extra-chromosomal arrays exhibit the characteristics of heterochromatin; they are enriched with H3K27me3 and H3K9me3 (YUZYUK *et al.* 2009; BESSLER *et al.* 2010; MEISTER *et al.* 2011), which are associated with inactive transcription. Moreover, the extra-chromosomal arrays are usually found at the periphery of a nucleus (TOWBIN *et al.* 2011).

Anecdotally, we noticed that animals carrying high-copy number transgenes inhibited *pha-4*(RNAi)-associated lethality. Therefore, we designed an experiment to test the effect of extra-chromosomal arrays on RNAi. The pRF4 plasmid, which encodes a dominant mutant collagen, is commonly used as a co-injection marker due to its distinct "Roller" phenotype (KRAMER *et al.* 1990; MELLO *et al.* 1991; EVANS 2006). We injected pRF4 plasmids mixed with sperm testis DNA into wildtype worms and crossed the resulting lines ("roller" lines) with *daf-12*(rh61rh411) to generate a *daf-12* mutant strain stably carrying the extra-chromosomal array composed of multi-copy pRF4 plasmids. Wildtype and *daf-12* mutant Rollers produced Roller progeny at a rate of 70% and 66%, respectively, due to the partial transmission of the array. We exposed Rollers and non Rollers (F1) from Roller parents (P0) to *pha-4* dsRNA and found that the viability of progeny (F2) from Rollers was 15 times higher than that of progeny from non Rollers (Fig. 4.3). Offspring of the *daf-12* mutants carrying pRF4 also showed two fold higher

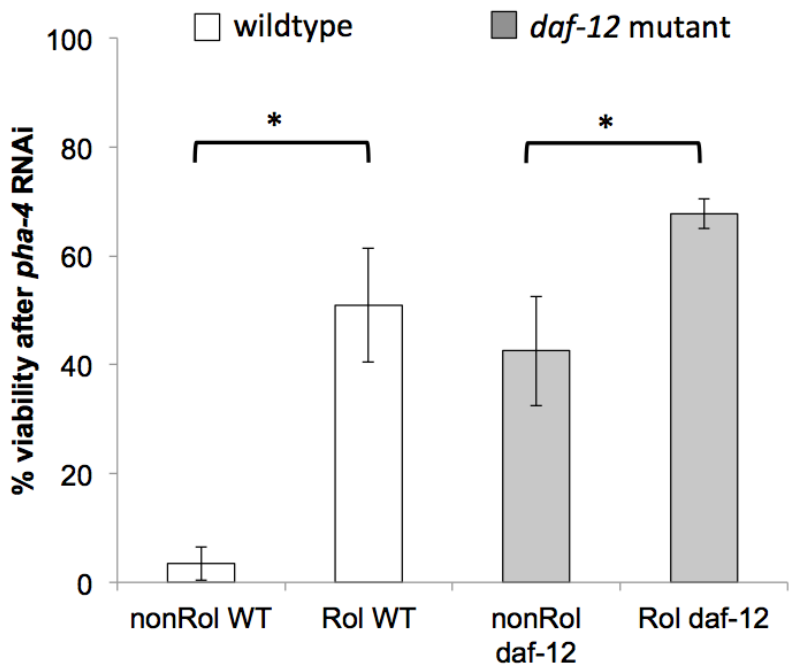


Figure 4.3. The presence of extra-chromosomal arrays repressed *pha-4* RNAi. Animals (Rol) carrying extra-chromosomal arrays that consisted of multiple copies of a pRF4 plasmid, which encodes a dominant mutant allele of *rol-6* (KRAMER *et al.* 1990; MELLO *et al.* 1991), were treated with *pha-4* RNAi. For comparison, animals that did not carry extra-chromosomal arrays (nonRol) were also treated with *pha-4* RNAi. The viabilities of their progeny were scored. n=3. *$p<0.05$ (Student t-test).

viability than progeny of non Rollers and *daf-12* mutants (Fig. 4.3), suggesting that the role of extra-chromosomal arrays in RNAi is in parallel with the role of DAF-12.

How can a multi-copy extra-chromosomal array inhibit *pha-4* RNAi? The observations that heterochromatin formation is implicated in transcriptional repression of the array (YUZYUK *et al.* 2009; BESSLER *et al.* 2010; MEISTER *et al.* 2011) and of a gene targeted by RNAi (GUANG *et al.* 2010; GU *et al.* 2012) may give us a clue. It is possible that the extra-chromosomal array titrates factors that are required for transcriptional inactivation away from the gene targeted by dsRNA, and as a consequence, the efficiency of RNAi is reduced. This argument would predict that the effect of a transgene on RNAi could be generalized to other genes targeted by the transcriptional RNAi pathway, not specifically to *pha-4*. Moreover, given that integrated arrays with a low-copy number are less likely to be silenced (PRAITIS *et al.* 2001; FRØKJÆR-JENSEN *et al.* 2008), transgenes may have an effect on RNAi in a copy-number dependent manner.

Transgenes encoding the wildtype version of an endogenous gene is commonly used to rescue mutant phenotypes. The effect of transgenes on RNAi described above indicates that one may need to be careful in drawing a conclusion from the experiments testing transgenic worms on RNAi, because the effect, if any, could come from the presence of transgene itself, independent of its sequence.

Older animals are more susceptible to RNAi than younger worms

As organisms age, their physiological functions deteriorate. Like vertebrates, *C. elegans* exhibits the characteristics of aging, such as reduction in muscle mass (HERNDON *et al.* 2002; EVASON *et al.* 2005), the decline in axon regeneration (BYRNE *et al.* 2014), and

altered sensitivity to sensory inputs (TSUI and VAN DER KOOY 2008). Young, wildtype *C. elegans* hermaphrodites produce viable embryos for 3-4 days (LUO *et al.* 2009), and even after reproduction ceases, they live for weeks (SHI and MURPHY 2013). To determine whether aging has an effect on RNAi sensitivity, we exposed worms of different ages (12hr, 24hr, 36hr, and 48hr-post-L4 stage) to *pha-4* RNAi for 12 hours at 25°C and examined the viability of the progeny (Fig. 4.4A). Interestingly, the viability of the progeny of older worms (36hr and 48hr-post-L4) produced was only about 1/4 of that of younger worms (Fig. 4.4B), indicating that RNAi was enhanced in the offspring from older worms. Like wildtype animals, progeny of older *daf-12* mutants exhibited higher *pha-4* RNAi-induced lethality than that of younger *daf-12* mutants, suggesting that DAF-12 is not engaged in the effect of aging in RNAi (Fig. 4.4C). *lin-26* RNAi produced a similar pattern, where RNAi was more effective in older animals than younger worms (Fig. 4.4D). These results suggest that the aging process may generally enhance RNAi.

Currently, it is not clear whether old adults or their progeny are responsible for enhanced RNAi phenotypes; it is possible that any physiological or molecular changes in aged worms increases the amount of silencing signals inherited in zygotes, thereby aggravating RNAi phenotypes in zygotes. Alternatively, zygotes of older worms may be highly sensitive to RNAi for unknown reasons. Further investigation would be required to identify the aging-related processes that increase the RNAi efficiency.

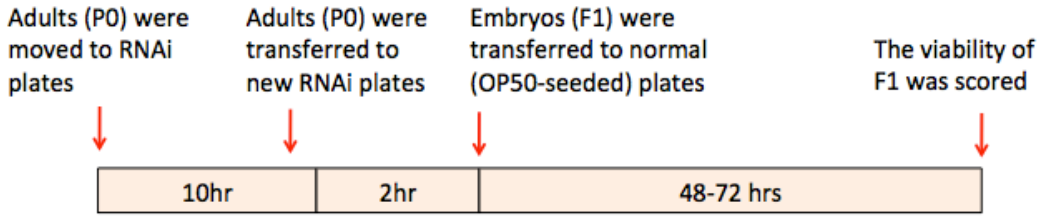
Disruption of neuronal network enhances RNAi sensitivity in embryos

C. elegans have twelve amphid neuron pairs whose ciliated endings are exposed to the environment (BARGMANN 2006). Among these chemosensory neurons, ASI neurons not

Figure 4.4. Aged animals increased the efficiency of feeding RNAi.

(A) A timeline of the experiment to test the effect of aging on the RNAi efficacy. Worms (P0) with different ages (12, 24, 36, and 48hr-post-L4) were treated with RNAi for 10hrs to purge any germ cells produced before the uptake of dsRNA and initiate RNAi. Then, the worms (P0) were transferred to new RNAi plates and their embryos (F1) were collected in two hours. Two days later, viabilities of F1 were scored. (B and C) Wildtype (B) and *daf-12*(rh61rh411) (C) were treated with *pha-4* RNAi and viabilities of their progeny were recorded. n=3. Error bars represent standard deviation. (D) Like *pha-4* RNAi, *lin-26* RNAi causes lethality. Wildtype worms with different ages were treated with *lin-26* RNAi and the numbers of their viable progeny were counted. n=3. Error bars represent standard deviation.

A



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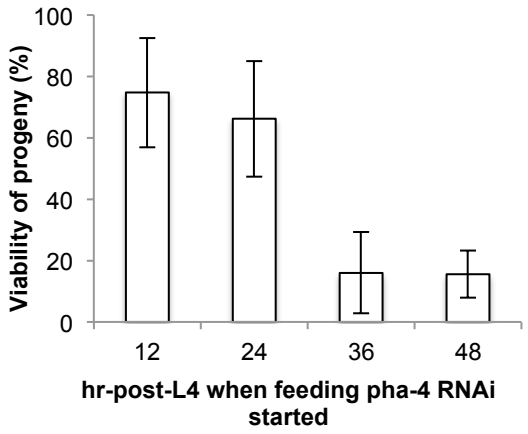

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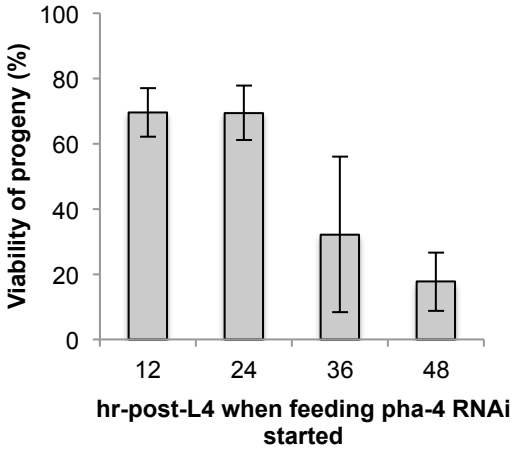

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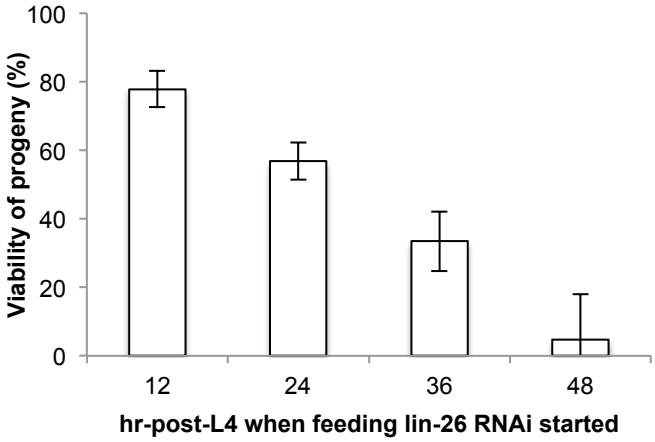


Figure 4.4 (continued). Aged animals increased the efficiency of feeding RNAi.

only sense temperature and certain chemicals (BARGMANN and HORVITZ 1991; BEVERLY *et al.* 2011), but also promote reproductive growth and dietary restriction-induced longevity, indicating that signals from ASI neurons can result in systemic changes throughout the organism (HUKEMA *et al.* 2006; BISHOP and GUARENTE 2007). To determine the role of environmental cues in RNAi, we decided to ablate the ASI neurons and test whether their absence altered RNAi efficiency. Many laser ablation studies have used transgenic worms that mark specific neurons to aid ablation. However, considering the aforementioned observation that the presence of a transgene itself changes RNAi sensitivity, we decided to avoid using any transgenic strains. Instead, we treated third stage larvae with DiD to label the amphid neurons (Fig. 4.5A and B) and ablated ASI neurons using laser (FANG-YEN *et al.* 2012). ASI neurons were identified by their position and morphology. After recovery and growth to the fourth larval stage, mock-ablated and ASI-ablated worms were treated with *pha-4* or *elt-2* dsRNA, which also causes lethality. We found that the progeny of ASI-ablated worms showed more severe RNAi phenotypes than control (mock-ablated) worms that were treated identically except for laser firing (Fig. 4.5C). The enhanced RNAi phenotype was not specific to ASI ablation because ablation of ASK chemosensory neurons positioned close to ASI neurons (Fig. 4.5B) also increased *pha-4* (RNAi) lethality (Fig. 4.5C). It is not yet clear whether the high sensitivity to RNAi is a phenotype produced by disrupted neuronal network or a specific feature of ASI and ASK ablation. However, preliminary data with ADL neuron ablation suggest that not all ablated neurons lead to enhanced RNAi (Fig. 4.5C). Of note, we cannot rule out the possibility that laser exposure itself during larval growth produces such effects.

Figure 4.5. ASI and ASK neuron ablation increased RNAi sensitivity in wildtype embryos.

(A) Positions of amphid neurons (lateral view; from neuronbank.org) (B) Amphid neurons were labeled with a fluorescent lipophilic dye DiI prior to ablation to identify ASI and ASK neurons. (C) The third larval stage worms were dye-filled, mounted on an agar pad and subjected to laser ablation. Control worms (mock-ablated worms) were treated the same way except for laser firing. When ablated worms and mock-ablated worms were recovered and grew to the fourth larval stage, they were treated with *pha-4* or *elt-2* RNAi and viability of their progeny was scored. Normalized viabilities of progeny from ASI-ablated worms (grey squares) and ASK-ablated worms (grey circles) after *pha-4* RNAi was significantly lower than 1 (dotted red line), suggesting that neuronal ablation or laser exposure caused enhanced *pha-4* RNAi phenotype. Moreover, progeny of ASI-ablated worms showed lower viability after *elt-2* RNAi treatment (white squares) than that of mock-ablated worms. ADL ablation (grey diamond) was performed as well, but due to a small sample size and a high variance, it was hard to draw any conclusion yet. n>3.

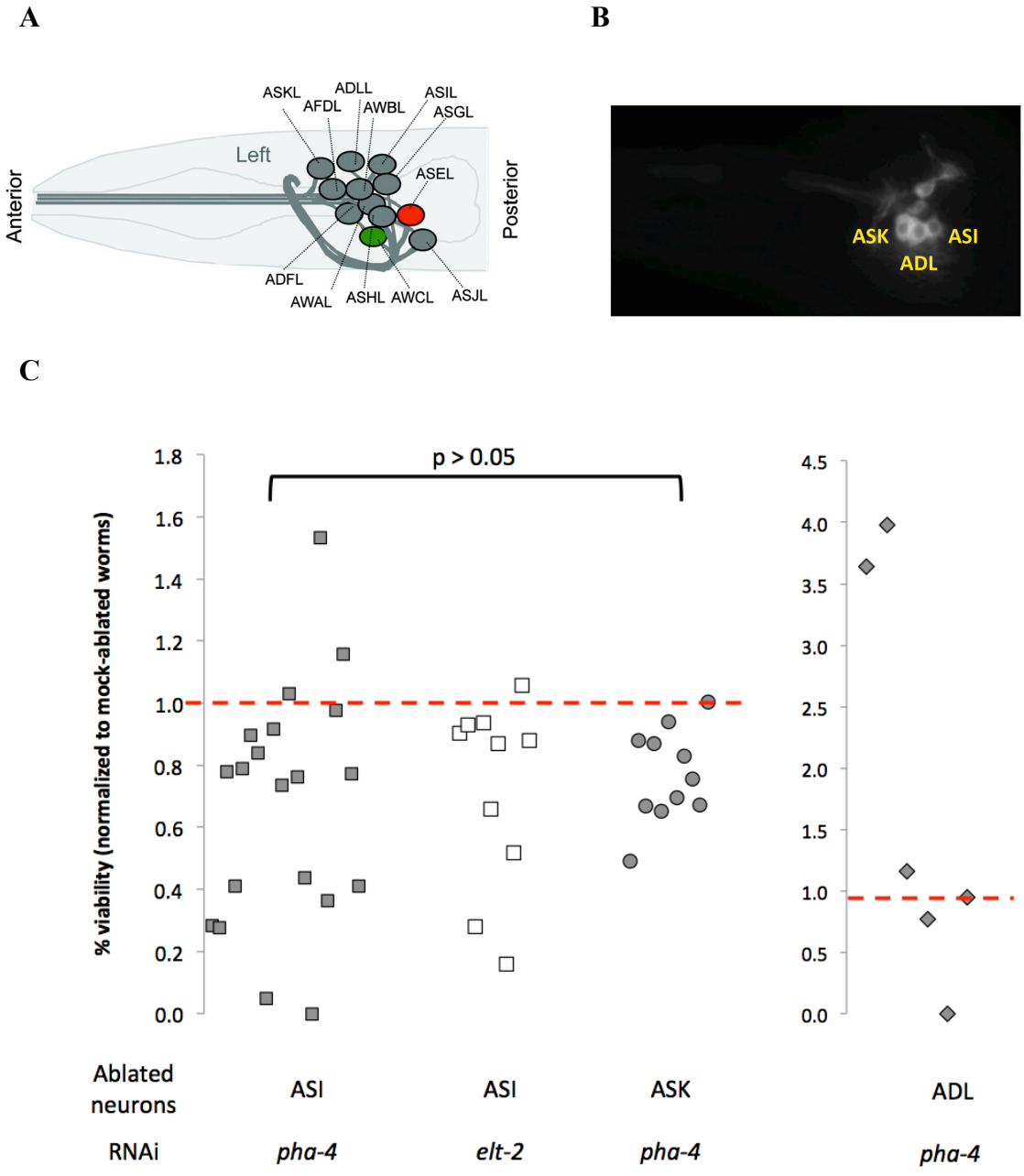


Figure 4.5 (continued). ASI and ASK neuron ablation increased RNAi sensitivity in wildtype embryos.

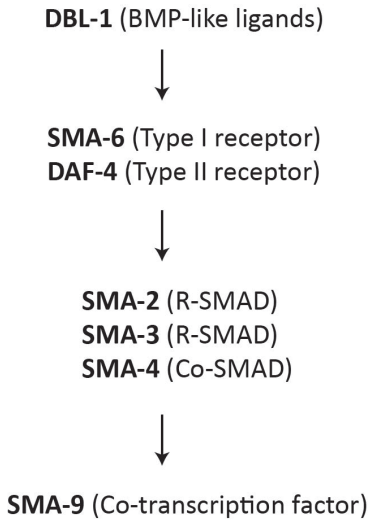
APPENDIX II

Additional Genes That Alter *pha-4*(RNAi) Lethality

In addition to TGF-beta pathway mutants, I tested worms carrying mutations in the BMP-like signaling pathway (also called DLB-1 pathway or SMA pathway) and *let-7* microRNA. BMP-like pathway is triggered by DBL-1, a *C. elegans* homologue of *Drosophila* decapentaplegic and the vertebrate bone morphogenetic proteins (SUZUKI *et al.* 1999). Active BMP-like pathway (Fig. 4.6A) regulates biological processes such as body size (SUZUKI *et al.* 1999; MORITA *et al.* 2002), innate immunity (MALLO *et al.* 2002; ZUGASTI and EWBANK 2009; ROBERTS *et al.* 2010), and metabolism (ROBERTS *et al.* 2010). Among five BMP-like pathway mutants I tested, *daf-4* and *sma-9* mutants showed strong *pha-4* RNAi response (Fig. 4.6B). As mentioned above, DAF-4 is a TGF-beta type II receptor acting in both TGF-beta dauer pathway and BMP-like pathway (ESTEVEZ *et al.* 1993; SAVAGE *et al.* 1996; MORITA *et al.* 1999; GUNTHER *et al.* 2000). Therefore, it would be interesting to know whether DAF-4 regulates *pha-4* RNAi via the TGF-beta dauer pathway, the BMP-like pathway, or both. SMA-9 is one of the transcription factors acting downstream of the BMP-like pathway (Fig. 4.6A) (LIANG *et al.* 2003; SAVAGE-DUNN *et al.* 2003). Interestingly, a microarray study revealed that some genes expressed in germ line are up-regulated in *sma-9*(wk55) mutants (LIANG *et al.* 2007). Feeding *sma-9* mutants with various RNAi food would help us determine whether SMA-9 is involved in RNAi in general or specifically interacts with *pha-4*.

Another mutant strain that showed strong *pha-4*(RNAi)-associated lethality was

A



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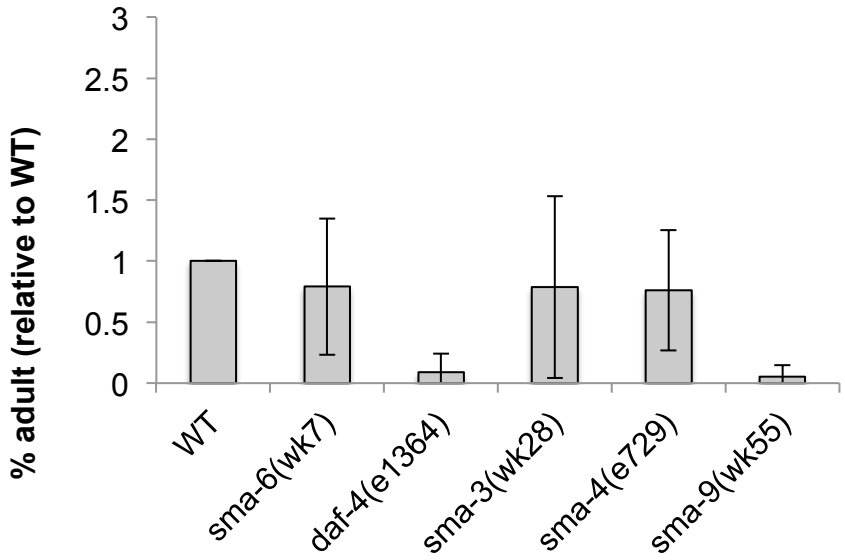

Figure 4.6. The BMP-like signaling pathway may regulate *pha-4* RNAi.

(A) The BMP-like pathway in *C. elegans* (modified from SAVAGE-DUNN 2005). (B) The BMP-like pathway mutants were treated with *pha-4* RNAi and the viabilities of their progeny were scored. n=3. Error bars represent standard deviation.

let-7(n2853) (Fig. 4.7). *let-7* is a well-conserved microRNA (PASQUINELLI *et al.* 2000) and plays a crucial role in developmental timing in *C. elegans* (REINHART *et al.* 2000). We were interested in *let-7* microRNA because it is a downstream target gene of DAF-12 (HAMMELL *et al.* 2009). Moreover, *daf-12* itself is a target of *let-7*, forming a negative feedback loop (GROßHANS *et al.* 2005; ZHANG *et al.* 2007; HAMMELL *et al.* 2009). Of note, it has been suggested that *let-7* targets *pha-4* and down-regulates *pha-4* expression in the intestine (GROßHANS *et al.* 2005). However, changes in the translation rate and degradation of *pha-4* transcripts were not observed in *let-7* mutants (DING and GROßHANS 2009). Therefore, down-regulation of *pha-4* expression by *let-7* is rather controversial.

To test whether *let-7* regulates *pha-4* RNAi, we grew *let-7*(n2853) temperature sensitive strains at the permissive temperature and treated them with *pha-4* RNAi at the restrictive temperature. Compared with N2 wildtype animals, *let-7* mutants exhibited stronger *pha-4* RNAi response (Fig. 4.7), which is the opposite behavior of *daf-12* null mutants. These opposing *pha-4* RNAi phenotypes of *let-7* and *daf-12* mutants would allow us to run epistasis analysis in the future to determine whether the relationship between DAF-12 and *let-7* described above is important in RNAi regulation.

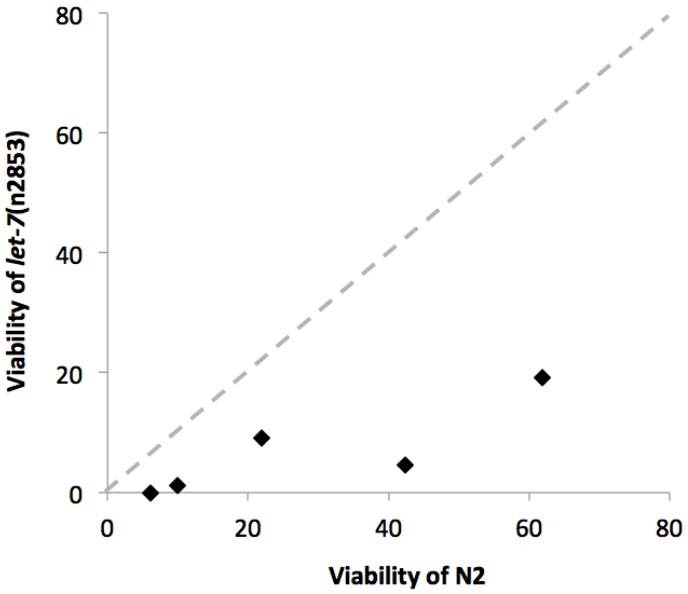

Figure 4.7. *let-7* mutants show robust *pha-4* RNAi

N2 (wildtype) and *let-7*(n2853; temperature sensitive) mutants were maintained at the permissive temperature and then treated with *pha-4* RNAi at the restrictive temperature. The grey dotted line was drawn on the graph to help compare the viability of *let-7* mutants to that of N2. n=2.

APPENDIX III

C. elegans strains used in this study

Table 4.1. Strain names and genotypes of C. *elegans* strains used in this study.

Strain Name	Genotype
AA1	*daf-12*(rh257) X
AA411	*din-1*(dh149) II
AA82	*daf-12*(rh284) X
AA83	*daf-12*(rh62rh157) X
AA88	*daf-12*(rh193) X
AA89	*daf-12*(rh274) X
CS1	*sma-9*(wk55) X
DR1369	*sma-4*(e729)III
DR1605	*daf-5*(m512) II
LT153	*sma-3*(wk28) III
LT186	*sma-6*(wk7) II
LT85	*sma-3*(wk20) III
MT7626	*let-7*(n2853) X
RB2589	*daf-3*(ok3610) X
SM2020	*daf-12*(rh61rh411) X
SM2021	Wildtype sibling of SM2020
SM2067	*smg-1*(cc546 ts)I ; *pha-4* (q500) *rol-9* (sc148)V; *daf-12* (rh61rh411)X
SM2068	*smg-1*(cc546 ts)I ; *pha-4* (q500) *rol-9* (sc148)V. WT sibling of SM2067
SM2076	*pha-4*(q500) *rol-9*(sc148)/ *fog-2*(q71) V; *daf-12*(rh61rh411) X
SM2077	*pha-4*(q500) *rol-9*(sc148)/ *fog-2*(q71) V. WT sibling of SM2076
SM2134	wildtype sibling of SM2135, SM2136m and SM2142
SM2135	*daf-12*(rh61rh411) X
SM2136	*daf-4*(e1364) III; *daf-12(*rh61rh411) X
SM2142	*daf-4*(e1364) III
SM2172	*smg-1*(cc546ts) I; *ergo-1*(tm1860) *pha-4*(zu225) V
SM2234	*smg-1*(cc546ts) I; *pha-4*(zu225) V. Wildtype sibling of SM2235.

Table 4.1 (continued). Strain names and genotypes of C. *elegans* strains used in this study.

Strain Name	Genotype
SM2235	*smg-1*(cc546ts) I; *pha-4*(zu225) V; *daf-12*(rh61rh411) X
SM2255	Wildtype sibling of SM2256, SM2257, SM2258
SM2256	*nhr-8*(ok186) IV
SM2257	*daf-12*(rh61rh411) X
SM2258	*nhr-8*(ok186) IV; *daf-12*(rh61rh411) X
SM2280	ltIs37 [*pie-1*p::mCherry::*his-58* (pAA64) + *unc-119*(+)]. Wildtype sibling of SM2281. Hermaphrodites and males
SM2281	*daf-12*(rh61rh411) X; ltIs37 [*pie-1*p::mCherry::*his-58* (pAA64) + *unc-119*(+)]. Hermaphrodites and males
SM2284	pxEx559 [*rol-6* (su1006) from PRF4]
SM2340	*daf-12*(rh61rh411) X; pxEx559 [*rol-6* (su1006) from PRF4]
YY158	*nrde-3*(gg66) X
YY186	*nrde-2*(gg91) II

APPENDIX IV

Primer sequences used in this study

Table 4.2. Primer sequences.

Target gene	Target region	Sequence	Role
hrde-1	exon 1/2	CGA CGT CAA AGA GAA GTG CAC C	NRDE pathway genes (RT-qPCR)
	exon 2	GGG CAT ATC CTT GAT GTC CAC TGG	
nrde-1	N/A	ACG CTA GAA GAG CTA ATC GAA GTG	
	N/A	GGG CTT TCT ACA GCC TCT CTG TC	
nrde-2	N/A	GTG GTA CTG GCA AAC ATA CAG GCC	
	N/A	CGA TTC GCG GAA GAC CAG ATT C	
nrde-3	exon 3/4	AAT CCA CCA ATC TAC ACG CGT GG	
	exon 4	GGA AAG TTC CCG ACG AAT AGA GC	
nrde-4	exon 11	GCG GAT GTA TCT CGA TCA GGC	
	exon 12	ACG CTT CAT TAA CAG CTG TAG ACC	
Y46H3C.7	N/A	CAG TTC TTA CGG TGT AGA GCA TCA C	NRDE pathway targets (RT-qPCR)
	N/A	CGT TCC TTC TTC TTC TGT TGT TCT C	
Y9D1A.1	N/A	TAA AGA CGA CGA AGA AGG ATC GG	
	N/A	CGT CGT CTT TAT TGT AGA TGT TTC CG	
C01A2.1	exon 1	TGA AGA AGA CGA GCT TCT GAA AG	
	exon 2	GAG CTT TAC TGT TTC TTG TGA CCA TTG	
bath-45	exon 5/6	TCT ATG TCT TAA AGC AGA TCC TCG C	HRDE-1 target genes (RT-qPCR)
	exon 6	CCA TAC ATG ACC TCC ACA AGA TTC TG	
F15D4.5	exon 3/4	AAA CCT CCG CCG ATG AGC ATC	
	exon 4	TGA TCA GTG GGT AGT AGT TTC TCT GG	
B0250.8	exon 1/2	TTC GTC TTT CTC CAG GTA ACC CAA T	
	exon 3	AGT GAA TTT GAT AAC TCC TGC GGC	
F35G12.2.2	exon 1/2	ACA ACT AAG CTG GCT CGT TAT GG	RT-qPCR reference gene
	exon 2	CAC TTC GAA ATC TAC TGG AGC TTG	

Table 4.2 (continued). Primer sequences

Target gene	Target region	Sequence	Role
pha-4	1st ATG	ACG AAG AGT CGT ATC AAG AGA CCT A	ChIP
	1st ATG	ATC AAT AAT GTC CTC ATC ACT GGA TGG	
	intron 4	TGA AAG CTC TAA ATA TTT CCC AAG CA	
	int 4/ex 5	GCC AGT GGT AAA ACC TGA ATG AGA T	
	exon 7	CAA TCC CAG AAT TTC CTG AAC AAC AC	
	intron 7	TAG TAG GGG GTC AAC AAA ACA TCG T	
	3'UTR	TTC AAC CGG CGT GCT TGT GT	
	3'UTR	GGC ATC TGG TAG AGG GGA ACC A	
eft-3	promoter	AGC GTT TTT CCT GTT CTC ACT GTT T	Pol II ChIP positive ctrl gene
	promoter	GAG TGC GGA CGG TAG AGA GAA TAA A	
elp-1	ex 8/int 8	CAC ACT TGC GAT GTT AAA TGG TTT AG	
	exon 9	CTA CCG AGT GGC ATG AAG ATT G	
srw-99	promoter	TCA AAA TGT TCC CAC GTC TAT AAA ACT TAC	Pol II ChIP negative ctrl gene
	promoter	AAA AAG AGC ATT TTG CAA TAC GTT AGA G	
pcaf-1	exon 1	CGA CGA CAA TTA ACA TAT TTC ACG TC	H3K9me3 ChIP ctrl gene
	int 1/exon2	GTT CGA CAG TCT GAA AAA GCA GAA A	

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